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Small RNAs that target G-rich sequences are generated by diverse biogenesis pathways in Epsilonproteobacteria

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Abstract

Bacterial small RNAs (sRNAs) are widespread post-transcriptional regulators that control bacterial stress responses and virulence. Nevertheless, little is known about how they arise and evolve. Homologs can be difficult to identify beyond the strain level using sequence-based approaches, and similar functionalities can arise by convergent evolution. Here, we found that the virulence-associated CJnc190 sRNA of the foodborne pathogen *Campylobacter jejuni* resembles the RepG sRNA from the gastric pathogen *Helicobacter pylori*. However, while both sRNAs bind G-rich sites in their target mRNAs using a C/U-rich loop, they largely differ in their biogenesis. RepG is transcribed from a stand-alone gene and does not require processing, whereas CJnc190 is transcribed from two promoters as precursors that are processed by RNase III and also has a *cis*-encoded antagonist, CJnc180. By comparing CJnc190 homologs in diverse *Campylobacter* species, we show that RNase III-dependent processing of CJnc190 appears to be a conserved feature even outside of *C. jejuni*. We also demonstrate the CJnc180 antisense partner is expressed in *C. coli*, yet here might be derived from the 3'UTR (untranslated region) of an upstream flagella-related gene. Our analysis of G-tract targeting sRNAs in Epsilonproteobacteria demonstrates that similar sRNAs can have markedly different biogenesis pathways.

KEYWORDS
Campylobacter jejuni, *Helicobacter pylori*, pathogenesis, RNase III, sRNA biogenesis

1 | INTRODUCTION

Many of the bacterial small RNAs (sRNAs) initially characterized were identified as curious transcripts dedicated to the control of mobile genetic elements such as plasmids (Waters & Storz, 2009). In the past decades, they have emerged as global *trans*-acting regulators of gene expression, and provide a layer of post-transcriptional control that is widespread, diverse in mechanism, and permeates all aspects of bacterial physiology (Hör et al., 2020; Kavita et al., 2018; Wagner & Romby, 2015). In pathogens, sRNAs regulate genes that affect stress responses and host interactions (Svensson & Sharma, 2016; Westermann, 2018). In principle, the canonical sRNA base-pairs near the ribosome binding site (RBS) of target mRNAs to control their stability or translation (Storz et al., 2011). In reality, sRNAs display a variety of regulatory mechanisms involving, e.g., activation of translation, interaction with coding regions, non-coding transcripts, and/or proteins, or even encoding regulatory proteins themselves (Carrier et al., 2018). Many, but not all, sRNAs require RNA chaperones, such as Hfq or ProQ, for their expression and activity (Holmqvist & Vogel, 2018; Kavita et al., 2018). While the

first *trans*-acting sRNAs in bacterial chromosomes were identified serendipitously via genetic screens or bioinformatic analysis of intergenic regions (Barquist & Vogel, 2015; Livny & Waldor, 2007; Vogel & Sharma, 2005), more recently RNA-seq approaches have exponentially expanded the catalog of bacterial sRNAs (Barquist & Vogel, 2015). Notably, this has revealed sRNAs that are embedded in diverse genomic contexts, such as processed from mRNA 3' untranslated regions (UTRs) or antisense to coding genes (Adams & Storz, 2020; Miyakoshi et al., 2015; Thomason & Storz, 2010).

Transcriptome analyses have also revealed a wealth of sRNAs in Epsilonproteobacteria such as the widespread human pathogens *Helicobacter pylori* and *Campylobacter jejuni* (Bischler et al., 2015; Dugar et al., 2013; Kreuder et al., 2017; Porcelli et al., 2013; Sharma et al., 2010; Taveirne et al., 2013). While *H. pylori* resides in the stomach of half the world's population and is associated with gastric disease, including cancer (Salama et al., 2013), *Campylobacter* is currently the most common cause of bacterial foodborne gastroenteritis worldwide (Havelaar et al., 2015). While most *Campylobacter*-associated gastroenteritis cases are caused by *C. jejuni*, 1%–25% can be attributed to its relative *C. coli* (Man, 2011). Homologs of sRNA chaperones such as ProQ or Hfq are absent in Epsilonproteobacteria and the mechanisms and functions of sRNAs in these bacteria are still largely unknown (Pernitzsch & Sharma, 2012; Quendera et al., 2020; Rieder et al., 2012). However, characterization of some candidate sRNAs has already uncovered new mechanisms of regulation and connections to phase-variable expression control (Pernitzsch et al., 2014, 2021), as well as revealed sRNAs involved in virulence (Eisenbart et al., 2020; Kinoshita-Daitoku et al., 2021; Vannini et al., 2016). For example, length variation of a G-tract in the 5'UTR of a chemotaxis receptor mRNA, which is targeted by a C/U-rich loop in the sRNA RepG (regulator of polymeric G repeats) modulates the outcome of post-transcriptional regulation by the sRNA in *H. pylori* (Pernitzsch et al., 2014). Recently, a nickel-regulated *H. pylori* sRNA, NikS, was identified as a phase-variable repressor of major virulence factors such as CagA, thereby affecting animal colonization and in vitro virulence-associated phenotypes (Eisenbart et al., 2020; Kinoshita-Daitoku et al., 2021). Therefore, *H. pylori* sRNAs provide an intersection between *H. pylori* strain variability, virulence, and gene regulation. Transcriptome maps of several *C. jejuni* isolates likewise revealed conserved and strain-specific sRNAs that might influence virulence (Dugar et al., 2013; Porcelli et al., 2013; Taveirne et al., 2013), and for some of these first functions have been reported (Kreuder et al., 2020; Le et al., 2015; Shen et al., 2016). For example, a set of sRNA paralogs is highly conserved in thermophilic *Campylobacter* species, and both their regulation by the σ^{28} motility-related sigma factor and base-pairing with flagella-related targets appears to also be highly conserved (Le et al., 2015). Moreover, we recently reported that the sRNA pair CJnc180/190 affects *C. jejuni* adherence and internalization in a tissue-engineered model of the human intestine (Alzheimer et al., 2020). CJnc190 represses expression of the colonization factor PtmG by binding to a G-rich sequence in the *ptmG* mRNA using a C/U-rich loop (Svensson & Sharma, 2021). CJnc190 is generated by a unique biogenesis pathway involving processing by RNase III and is also antagonized by its *cis*-encoded sRNA partner, CJnc180.

Despite their central role in gene regulation, very little is known about how sRNA genes arise, evolve, and are turned over (Dutcher & Raghavan, 2018; Gottesman & Storz, 2011; Updegrove et al., 2015). This is in part due to difficulties in their identification based on homology (Lindgreen et al., 2014), meaning only a handful of highly conserved examples encoded in intergenic regions have been compared. Most appear to be relatively new inventions and are also exemplified by rapid evolution (Gottesman & Storz, 2011; Jose et al., 2019). Some key features can mark a functional sRNA, such as an environmentally regulated promoter, a Rho-independent terminator, structured regions that provide stability, accessible seed regions, or RNA chaperone binding motifs (Updegrove et al., 2015). While initial screens based on comparative genomics identified sRNAs as conserved blocks in empty intergenic regions, building homologous groups of these regulators in more distantly related species can be challenging. Identification and analysis of homologous sRNAs in diverse species can provide insight into their functions and mechanisms. So far, only a handful of systematic studies of bacterial sRNA evolution are available (Cerutti et al., 2017; Kacharia et al., 2017; Peer & Margalit, 2014; Raghavan et al., 2015; Skippington & Ragan, 2012). These often focus on stand-alone sRNAs with relatively straightforward biogenesis pathways and exclude those hidden in diverse genomic contexts more recently revealed by RNA-seq approaches (Adams & Storz, 2020).

Here, we examined the conservation and biogenesis pathways of a set of Epsilonproteobacterial sRNAs that target mRNAs using unique, extended C/U-rich stretches in single-stranded loops. We identified CJnc190 homologs by homology searches and/or inspection of similar genomic contexts in the genome sequences of several *Campylobacter* species and showed that repression of the cognate *ptmG* gene by the *C. coli* sRNA is conserved. Sequence analysis of putative CJnc190 precursors suggests that the processing of CJnc190 by RNase III is also conserved in *Campylobacter* species, suggesting it has a key regulatory function. However, in *C. coli*, only one of the two CJnc190 promoters that we identified in *C. jejuni* appears to be conserved. The *cis*-encoded antagonist of CJnc190, CJnc180, is expressed in *C. coli* despite the absence of the *C. jejuni* CJnc180 promoter sequence. We provide evidence to support the transcription of CJnc180 in *C. coli*, and identification of its 5' end in *C. coli* suggests that the antisense RNA is derived from the 3'UTR of the upstream flagella-related mRNA. Finally, we show that *C. jejuni* CJnc190 and *H. pylori* RepG share a similar C/U-rich targeting loop. Thus, they likely share similar functions and mechanisms of action, despite limited overall sequence similarity, different characterized mRNA targets, and distinct biogenesis pathways with markedly different levels of complexity. Overall, we show that sRNAs with similar activities can have biogenesis pathways of vastly different complexity, and propose that these differences might evolve to suit the environment of the bacterium.

2 | RESULTS

2.1 | *C. jejuni* CJnc190 and *H. pylori* RepG share a C/U-rich targeting loop

In our recent work, we showed that processed CJnc190 encoded in the CJnc190/180 sRNA locus of *C. jejuni* strain NCTC11168 represses *ptmG* translation by base-pairing with its G-rich translation initiation region using a single-stranded C/U-rich sequence within its second stem-loop (SL2; Figure 1a; Svensson & Sharma, 2021). We noticed that the mature CJnc190 sRNA resembles the *H. pylori* sRNA RepG, which also uses a C/U-rich loop to bind to a G-rich sequence in the 5'UTR of its target mRNA *tlpB*-HP0102 (Pernitzsch et al., 2014, 2021), in structure and function. Although the two sRNAs regulate distinct genes (*ptmG* and *tlpB*) in their endogenous hosts that are not shared by the two species, they both have a two stem-loop format with a similar C/U-rich SL2 that base-pairs with their respective targets (Figure 1b,c). They are also encoded in different genomic contexts with different flanking genes and are generated by distinct biogenesis pathways. RepG is encoded as a stand-alone sRNA that is transcribed from a single promoter and does not require processing for its expression or stability (Pernitzsch et al., 2014). In contrast, CJnc190 is transcribed as precursors from two promoters (P1 & P2), which are then processed by RNase III into the mature, stable, and/or active form of the sRNA (Svensson & Sharma, 2021; Figure 1a,b). In addition, an antisense RNA, CJnc180, is transcribed from the opposite strand of CJnc190 and antagonizes its regulation of *ptmG*.

To further examine the extent of this similarity in structure and loop sequence between CJnc190 and RepG, we compared putative CJnc190 and RepG homologs from additional *Campylobacter* and *Helicobacter* strains or species. We identified putative CJnc190 homologs in *C. jejuni*, *C. coli*, and *C. hepaticus* using the mature, active sRNA sequence from *C. jejuni* strain NCTC11168 and either the homology/structure tool GLASSgo (Lott et al., 2018) or manual inspection of regions adjacent to homologs of the genes flanking the sRNAs (Cj1650 and Cj1651c/*map*). We used GLASSgo and the RepG sequence of *H. pylori* strain 26695 to identify putative RepG homologs in additional *H. pylori* isolates, in *H. mustelae*, as well as *H. cetorum*. CMfinder covariance analysis (Yao et al., 2006) of several unique putative CJnc190/RepG sRNA sequences from *Campylobacter*/*Helicobacter* (Figure 1d), as well as inspection of the predicted secondary structures of selected unique sequences from *C. coli* and *C. hepaticus* (Figure S1a,b) showed a general conservation of the SL1-SL2 structure despite only limited overall sequence similarity. Most strikingly, this showed that these *Helicobacter* and *Campylobacter* sRNAs share an extended single-stranded C/U-rich region in SL2, with almost perfect homology of a CCUCCCCCU motif in the loop region that is used by the *C. jejuni* NCTC11168 and *H. pylori* 26695 homologs to base-pair with their respective target mRNAs (Figure 1b,c).

2.2 | RepG and CJnc190 bind G-rich sequences in target mRNAs

The RepG sRNA uses its C/U-rich SL2 to pair with a phase-variable homopolymeric G-stretch in the *tlpB*-HP0102 mRNA leader (Figure S2a), which varies in length between different *H. pylori* strains (Pernitzsch et al., 2014, 2021). In contrast, CJnc190 targets an imperfect G-rich sequence over the *ptmG* RBS in *C. jejuni* NCTC11168 (Figures 1a and 2a). We next sought to determine if the *ptmG* targeting site used by CJnc190 might be, like for the RepG-target *tlpB*, variable between *Campylobacter* isolates. The regions upstream of most *ptmG* homologs in *Campylobacter* included several G-residues clustered near the RBS (Figure 1d,e). Some *ptmG* homologs instead had a potentially phase-variable homopolymeric G-tract of different lengths at the 3' end of the upstream gene. For example, in *C. jejuni* IA3092 and *C. coli* RM1875, the position and length of the G-repeat could determine if the translation of the upstream gene either reaches an in-frame stop codon to generate two separate ORFs (open reading frames) (the upstream gene and *ptmG*), or generates a fusion of the two genes via read-through of an out-of-frame stop codon into the downstream *ptmG*-homologous region (if the two genes are on the same transcript; Figure S1c). Base-pairing predictions between *C. coli* CJnc190 homologs and the G-rich translation initiation regions of their *ptmG* counterparts showed that regulation might be conserved (Figure S1a). Moreover, genome-wide in silico predictions for CJnc190 targets in *C. jejuni* NCTC11168 identified several mRNAs with G-rich sequences as potential targets (Table S1). The list of predicted CJnc190 targets did not include a *tlpB* homolog, or any chemotaxis receptor, indicating that CJnc190 and RepG have distinct targets in *C. jejuni* and *H. pylori*. However, several predicted CJnc190 targets are encoded in the flagellin glycosylation island of NCTC11168, like *ptmG*, or the capsule locus, including those with phase-variable homopolymeric G-stretches in coding regions, suggesting that CJnc190 might target G-repeats, similar to RepG (Figure S4 and Table S1). Overall, conservation analysis indicated that CJnc190 and RepG homologs are widespread among Epsilonproteobacteria. These sRNAs share conserved C/U-rich loops that are used to target G-rich sequences or homopolymeric G-repeats in target mRNAs, suggesting that they could either be evolutionarily related or have arisen by convergent evolution to serve a similar mechanistic function.

2.3 | *H. pylori* RepG can directly repress the *C. jejuni* CJnc190-target *ptmG*

We hypothesized that despite limited sequence homology and markedly different biogenesis pathways (Figure 1b,c), CJnc190 and RepG might be functionally or mechanistically similar sRNAs since they both target G-rich sequences. In line with this, the RepG C/U-rich loop was predicted to interact with similar residues of the *ptmG* RBS

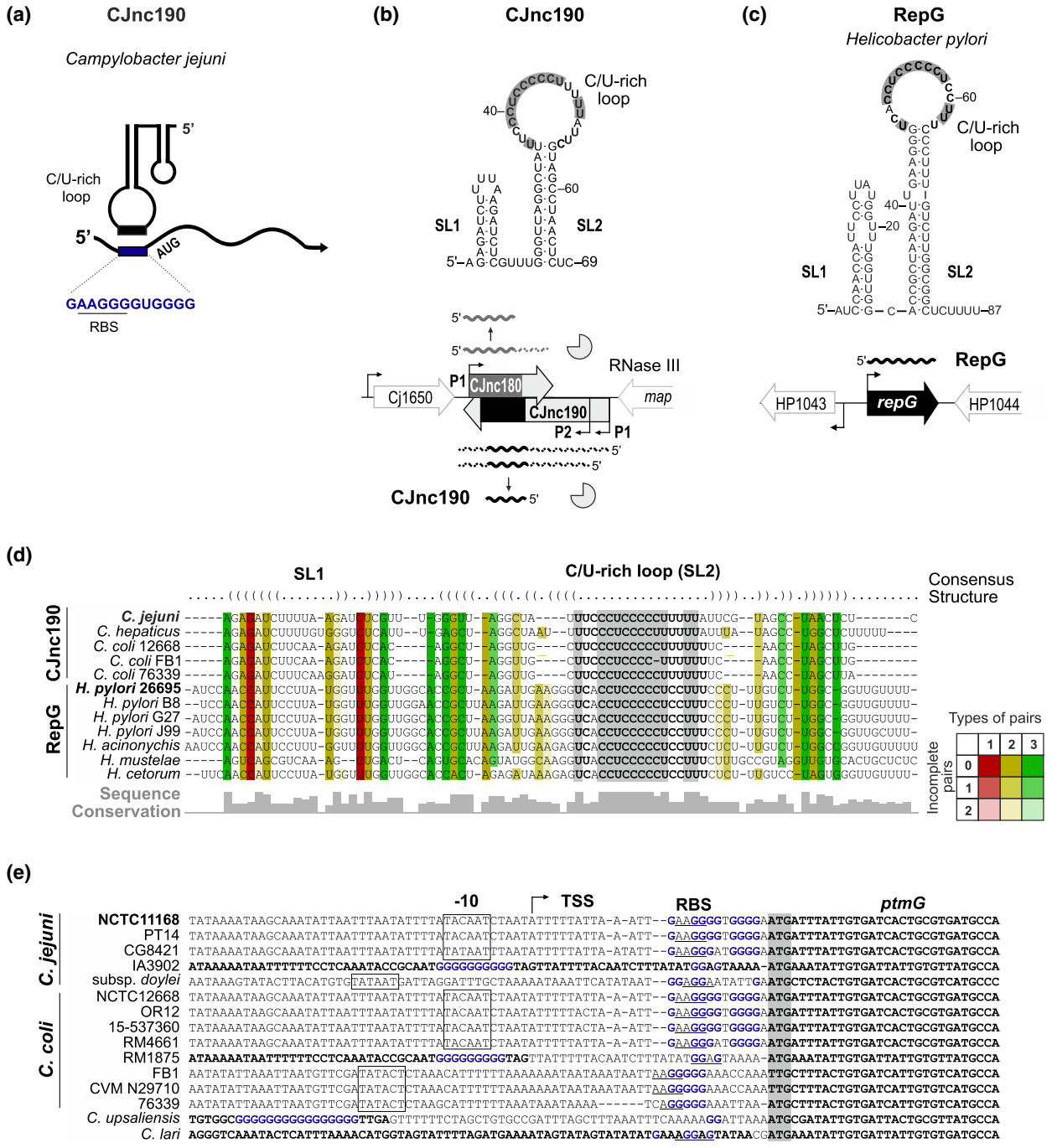


FIGURE 1 *C. jejuni* CJnc190 and *H. pylori* RepG share a C/U-rich loop that targets G-rich sequences. (a) CJnc190 from *C. jejuni* strain NCTC11168 uses a C/U-rich loop to repress PtmG expression via base-pairing with the G-rich RBS of *ptmG* mRNA (Svensson & Sharma, 2021). (b,c) Secondary structure, genomic context, and biogenesis of *C. jejuni* CJnc190 and *H. pylori* RepG (Pernitzsch et al., 2014; Svensson & Sharma, 2021). (Left) CJnc190 is transcribed as precursors from two promoters (P1/P2) and is independently processed by RNase III. Its *cis*-encoded antagonist CJnc180 is processed by RNase III in a CJnc190-dependent manner. (Right) RepG is transcribed from a stand-alone gene and not further processed. SL1/SL2: stem-loops. Bold: SL2 pyrimidines. Grey: identical residues (see panel d). Arrows: TSS. (d) Alignment and secondary structure motifs of *C. jejuni* CJnc190 and CJnc190/RepG homologs from *Campylobacter*/*Helicobacter* generated by CMFinder (Yao et al., 2006). Grey: SL2 >90% conserved residues. Colors: the number of base-pair types observed. Depth of color: number of incompatible pairs. Sequence conservation: grey bars below the alignment. (e) Alignment of *ptmG* upstream regions. Bold: coding nucleotides. Blue: G-rich regions. Putative promoters (based on the NCTC11168 −10 box), TSS, *ptmG* start codons, and RBS are indicated

targeted by CJnc190 (Figure 2a). Reciprocal predictions suggested that CJnc190 could also base-pair with the poly(G) tract of *tlpB*-HP0102 mRNA from *H. pylori* 26695 (12Gs; Figure S2b). We next determined if RepG could rescue repression of *ptmG* in vivo in the absence of CJnc190. Deletion of CJnc180/190 upregulates a PtmG-3×FLAG translational fusion at the protein and mRNA level, which

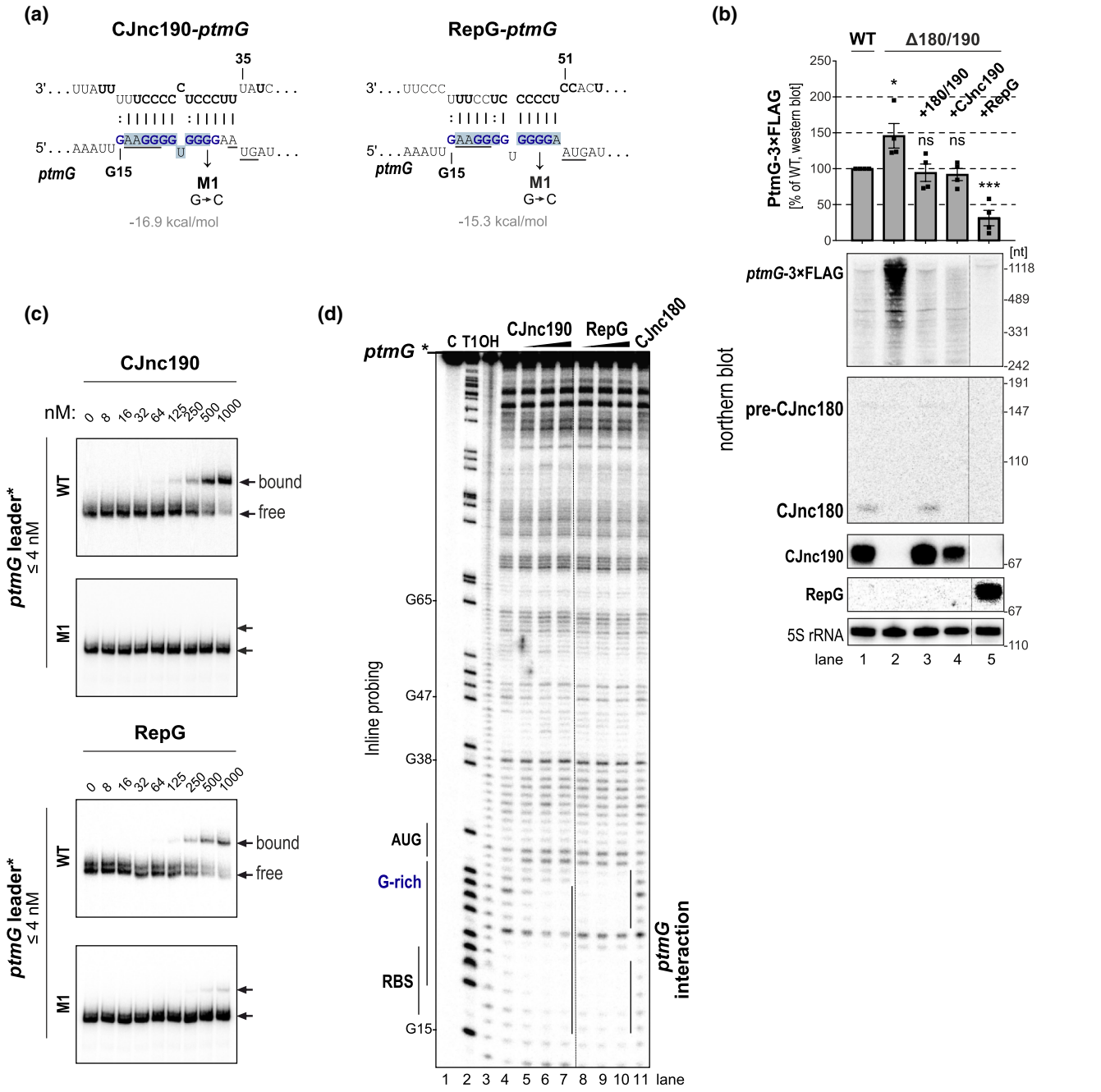


FIGURE 2 Both *C. jejuni* CJnc190 and *H. pylori* RepG can repress *C. jejuni ptmG*. (a) Validated/predicted base-pairing between CJnc190/RepG sRNAs and the *ptmG* leader. M1: single G-C exchange. Bold: C/U residues of loop 2. Grey highlighting: shared CJnc190/RepG residues. Blue residues: *ptmG* Gs. Underlined: *ptmG* RBS/start codon. Blue highlighting: *ptmG* bases protected by CJnc190 or RepG in Inline probing (panel d). The CJnc190-*ptmG* interaction is based on (Svensson & Sharma, 2021). (b) Regulation of PtmG levels in *C. jejuni* by CJnc190 or RepG. A 3×FLAG tag was fused to the penultimate codon of *ptmG* at its native locus in *C. jejuni* NCTC11168 Δ180/190. CJnc180/190, CJnc190 only, or RepG from *H. pylori* 26695 were expressed in *trans* from their native promoters at *rdxA*. Total protein was analyzed by western blotting with an anti-FLAG antibody. (*) $p < .05$, (***) $p < .001$, (ns) not significant (Student's *t* test, $n = 4$). See Figure S4 for a representative western blot. (c) In vitro gel mobility shift assays of CJnc190 and RepG with 5'-end radiolabeled *ptmG* leader/5'-coding region (204 nt transcript). Free *ptmG* and *ptmG*-sRNA bound complexes were resolved by gel electrophoresis under native conditions. M1: G-C exchange in *ptmG* (panel a). (d) Inline probing of CJnc190 and RepG base-pairing with the *ptmG* 5'UTR in vitro. Radiolabeled (5' end, 2 pmol) *ptmG* was incubated with unlabeled CJnc190/RepG (2, 20, or 100 pmol), or negative control CJnc180 (100 pmol) under mild alkaline conditions. Cleavage products were analyzed on a denaturing polyacrylamide gel. Dashed line: cropping of intervening lanes. C, untreated control; T1 ladder, G residues (indicated on left); OH, all positions (alkaline hydrolysis)

can be complemented in *trans* at the neutral *rdxA* locus by CJnc190 alone (without CJnc180; Svensson & Sharma, 2021). We introduced RepG from *H. pylori* strain 26695, under control of its native promoter, into *rdxA* of a *C. jejuni* NCTC11168 *ptmG*::3×FLAG tagged and ΔCJnc180/190 (Δ180/190) deletion background. Western and northern blot analysis showed that CJnc180/190, CJnc190 alone, or *H. pylori* RepG could repress *ptmG*-3×FLAG protein and mRNA levels in the Δ180/190 background (Figure 2b and Figure S3). In fact, PtmG-3×FLAG levels were even lower in the strain expressing RepG compared to the wild-type or CJnc190 complemented strains.

We next validated that RepG base-pairs with the *ptmG* G-rich RBS like CJnc190. In vitro-transcribed, 5' end radiolabeled *ptmG* bound both CJnc190 and RepG in gel mobility shift assays (Figure 2c). Moreover, a single G-to-C nucleotide (nt) exchange in *ptmG* in the predicted RepG interaction site (M1, Figure 2a) was sufficient to nearly abolish binding. Inline probing (Regulski & Breaker, 2008) with the same 5' end-labeled *ptmG* leader confirmed bases participating in the *ptmG*-RepG interaction (Figure 2d). Mobility shift assays with 5' end-labeled CJnc190 showed that, consistent with interaction predictions, CJnc190 can bind the *tlpB* leader in vitro (Figure S2c). Despite several attempts, we could not express CJnc190 in *H. pylori* to appreciable levels to determine if the *C. jejuni* sRNA could regulate *tlpB* even from the RepG promoter (data not shown), suggesting that CJnc190 might be unstable in *H. pylori*. Altogether, in silico, in vitro, and in vivo observations suggest that CJnc190 and RepG might use a similar regulatory mechanism to target G-rich sequences in mRNAs.

2.4 | CJnc190 and *ptmG* homologs are differentially conserved in *Campylobacter*

Next, we examined the conservation and absence/presence of CJnc180/190 homologs and the *ptmG* target in different *Campylobacter* species (Figure 3a). The CJnc190 target *ptmG* is a part of the strain-specific legionaminic acid biosynthesis pathway and is encoded in a strain-specific region of the *Campylobacter* flagellin glycosylation island (Figure S4a). While *C. coli* and *C. jejuni* are distinct species sharing on average 85% nucleotide identity, some *C. coli* isolates are apparently adapting to agricultural niches by the acquisition of DNA (up to 23% of their genomes) from *C. jejuni* to generate an apparently "hybrid" lineage (Sheppard et al., 2008, 2013). The *ptmG* gene is only sporadically conserved in *C. jejuni* and *C. coli*, but also detected in *C. upsaliensis* and *C. lari* isolates (Figure 3a; Champion et al., 2005; Howard et al., 2009). Moreover, its amino acid sequence relationship does not always follow phylogenetic relationships, supporting its horizontal gene transfer (Figure 3a). We wondered if CJnc190 might show a similar distribution. Above, we identified CJnc190 homologs in *C. jejuni*, *C. coli*, and *C. hepaticus* strains, but not in more distant species (Figure 1d). Although several strains lack the *ptmG* target, CJnc190 homologs were detected in all *C. jejuni* and *C. coli* isolates inspected (Figure 3a and Figure S5), indicating that CJnc190 likely regulates other targets in these strains. The CJnc190 homologs were always encoded in the same intergenic region, downstream of Cj1650 [encoding an RpoN-dependent FliS co-chaperone (Chaudhuri et al., 2011; Figure 3b)]. We did not detect CJnc190 in more distant species such as in *C. lari* or *C. upsaliensis* by homology searches, manual inspection, or from published RNA-seq data for *C. lari* (Riedel et al., 2020), although these species have potential *ptmG* homologs. In line with the observed lack of a CJnc190 homolog, the *C. upsaliensis* and *C. lari ptmG* translation initiation regions lack a significant G-rich character (Figure 1e).

The sequence similarity of CJnc190 homologs within species supported vertical transmission of the sRNA as part of the core *C. jejuni*, *C. coli*, and *C. hepaticus* genomes (Figure 3a). However, we observed two striking divergences from this theme. First, the CJnc190 sequence was not detected in the *C. jejuni* subsp. *doylei* genome (Dugar et al., 2013), and closer inspection suggested that the region downstream of Cj1650, encoding a FliS co-chaperone, in this subspecies no longer carries most of the nucleotides encoding CJnc180/190 (Figure 3b and Figure S5). Second, we detected evidence for horizontal transfer of CJnc190. In most *C. coli* isolates analyzed, CJnc190 showed ~75% nt identity with its *C. jejuni* counterpart, roughly in line with the overall nucleotide identity reported for the two species (Sheppard et al., 2013). Strikingly, the sequence of the sRNA in RM4661 was identical to that of *C. jejuni* (100% nt ID; Figure 3a). The phylogenetic relationship of the examined strains (based on *gyrB*) confirmed that RM4661 is a *C. coli* isolate, suggesting that RM4661 has acquired CJnc190 (and *ptmG*) via horizontal gene transfer (HGT) from *C. jejuni*.

We recently reported that CJnc180, the antisense partner of CJnc190, is transcribed from a single promoter in *C. jejuni* NCTC11168, while CJnc190 is transcribed as precursors from two promoters (Svensson & Sharma, 2021). Sequence alignments showed that the CJnc180 promoter is conserved in *C. jejuni* strains as well as *C. hepaticus* and is even present in *C. jejuni* subsp. *doylei*, which lacks most of the sRNA region (Figure 3b and Figure S5). In contrast, the CJnc180 promoter was not found to be conserved in *C. coli* (except for *C. coli* RM4661), making it unclear if and how it might be expressed. In addition, only the CJnc190 promoter P2 appears to be conserved in *C. coli* (Figure S5). Overall, our observations suggest that CJnc190 is generally part of the core genome of *C. jejuni*, *C. hepaticus*, and *C. coli*, but integrates variable *ptmG* into its regulon. Moreover, variations in promoter conservation were observed for both sRNAs among different strains and species.

2.5 | CJnc180 and CJnc190 expression and *ptmG* repression are conserved in *C. coli*

To validate our in silico predictions, we examined the expression and regulatory activity of CJnc190 in *C. coli*. We analyzed the total RNA from the *C. coli* isolate NCTC12668 (wild-type and isogenic Δ180/190) with a probe for the putative C/U-rich SL2. This detected a ~65 nt version of CJnc190, and unlike in *C. jejuni*, also detected a ~150 nt-long CJnc190 precursor (Figure 4a). The length of this putative precursor is in agreement with transcription from the single conserved P2

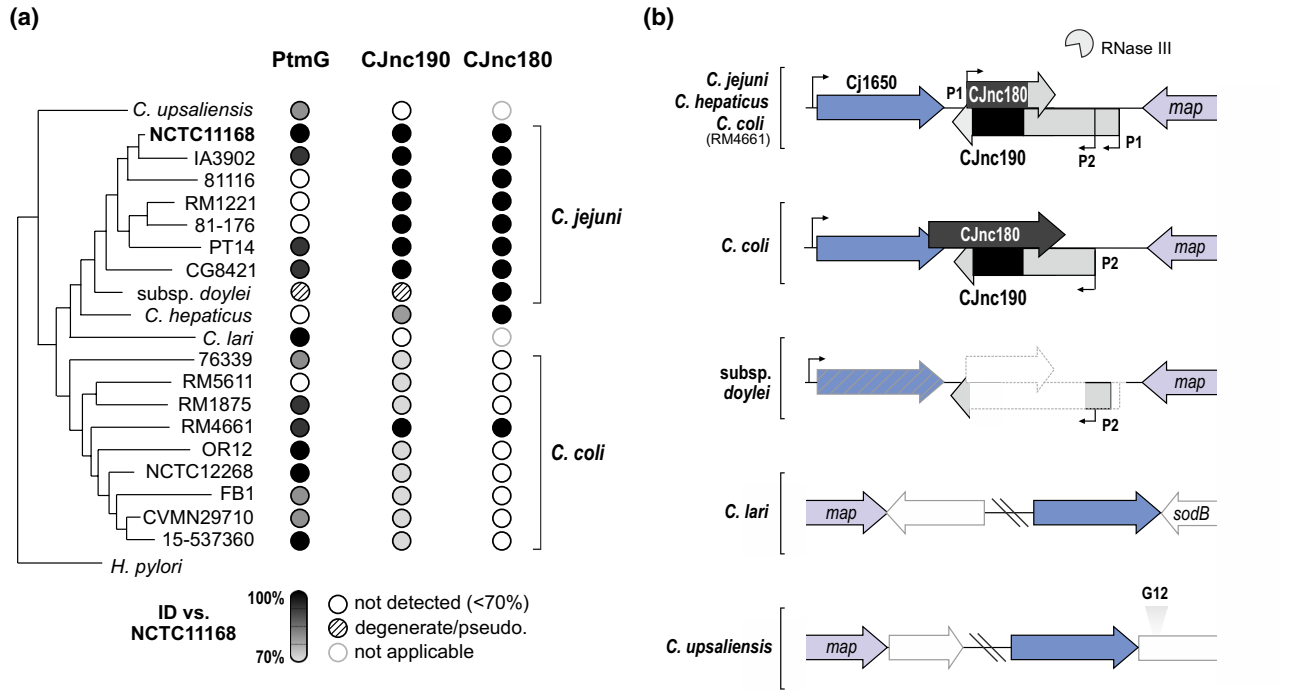


FIGURE 3 Variable conservation of CJnc180/90 and *ptmG* in *Campylobacter* species. (a) Detection and sequence homology of *ptmG* and CJnc180/190 in *Campylobacter* strains. The amino acid (aa, PtmG) or nucleotide (nt, CJnc190) sequences from *C. jejuni* NCTC11168 were used for tblastn or blastn searches, respectively. Shaded circles: % nt or aa identity (ID). CJnc180 promoters were detected by conservation of the NCTC11168 -10 box sequence. Empty circles (black outline): a homolog was not detected. Striped circles: sections of sequence are missing (CJnc180/190) or the homolog is a pseudogene (*ptmG*). For IA3902, only the aa sequence with homology to PtmG from an annotated fusion protein (see also Figure S1c) was used. Strain relationships (cladogram, left) are based on *gyrB* nucleotide sequences and were generated at phylogeny.fr (Dereeper et al., 2008). *H. pylori* strain 26695 was used for the comparison. Percent identities are also provided in Table S5. (b) Synteny and genomic context of three CJnc180/190 paradigms identified in *Campylobacter*. Arrows: potential TSS based on conserved *C. jejuni* NCTC11168 promoter −10 sequences. Dashed outlines: absent sequence. Hatched arrow: pseudogene. For extended alignments, see Figure S5. For *C. lari* and *C. upsaliensis*, sRNA sequences were not detected, even though these species encode homologs of the flanking genes from *C. jejuni* and *C. coli* at distinct genomic locations. *map*: methionine aminopeptidase. MCP: methyl-accepting chemotaxis protein. DUF1972: protein with a domain of unknown function

promoter in *C. coli* (Figure S5). Despite lack of conservation of the *C. jejuni* promoter sequence, the expression of a putative CJnc180 sRNA encoded antisense to CJnc190 was validated by northern blotting in our *C. coli* strain. However, only a single band of ~150 nt was detected, unlike the two CJnc180 bands corresponding to precursor and RNase III-dependent mature form in *C. jejuni* (Svensson & Sharma, 2021).

The expression of CJnc190 in *C. coli* suggested its function might be conserved. We mapped the 5' end of the mature sRNA and used this, together with its apparent northern blot length, to predict its secondary structure (Figure 4b,c). This showed that the canonical SL1-SL2 structure of CJnc190/RepG is conserved in *C. coli* (Figure 1b,c). Moreover, in silico predictions suggested that the *C. coli* sRNA could hybridize with the 5'UTR of its cognate *ptmG* (Figures 1d and 4c). Interestingly, both the amino acid and 5'UTR sequence of *C. coli* NCTC12668 *ptmG* are similar to that of *C. jejuni* NCTC11168, including the G-rich RBS (Figures 1e and 3a). In contrast, its CJnc190 counterpart shows overall only 76% nt ID to the NCTC11168 sRNA. We next determined if *C. coli* NCTC12668 CJnc190 could regulate its respective *ptmG* homolog by measuring mRNA levels by northern blotting, which are strongly repressed by CJnc190 in *C. jejuni* NCTC11168 (Svensson & Sharma, 2021). We observed that *ptmG* mRNA is strongly upregulated in the Δ180/190 mutant in both *C. jejuni* and *C. coli* (Figure 4a), validating that CJnc190 from *C. coli* has a similar function in *ptmG* regulation.

2.6 | Extended duplex structure substrates for RNase III processing are conserved among CJnc190 precursors

Our previous work demonstrated that CJnc190 precursors in *C. jejuni* are processed by RNase III. Surprisingly, the processing of CJnc190 is independent of the CJnc180 antisense RNA (asRNA) and instead dependent on an intramolecular duplex formed by sequences flanking the mature sRNA (Figure 5a; Svensson & Sharma, 2021). We next sought to explore if this intramolecular processing of CJnc190 by RNase III might be conserved outside of *C. jejuni*. We noticed that one of the two CJnc190 promoters from *C. jejuni* (promoter P2), which drives expression of pre-CJnc190 precursors and is approximately 70 nt upstream of the 5' end of the mature sRNA, is strongly conserved in *C. coli* (Figure S5). Alignment of the putative P1/P2 promoter regions from *Campylobacter* CJnc180/190 homologous

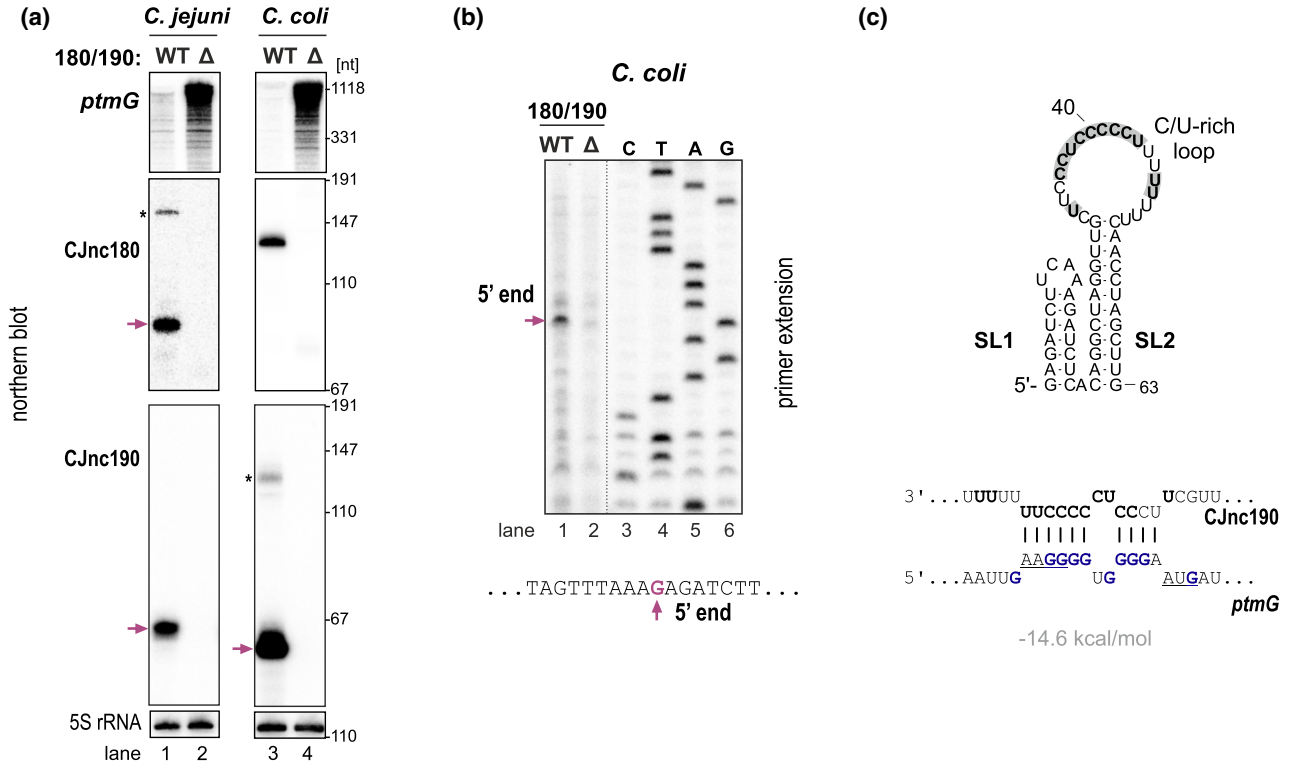


FIGURE 4 CJnc180 and CJnc190 homologs are expressed in *C. coli* strain NCTC12668. (a) Northern blot validation of CJnc180 and CJnc190 expression in total RNA from *C. coli* NCTC12668 versus *C. jejuni* NCTC11168 (wild-type and Δ180/190 backgrounds). The same blot was probed sequentially with strain-specific probes binding the mature sRNAs as follows: *C. jejuni* CJnc180/190 – CSO-0189/0185; *C. coli* CJnc180/190 – CSO-3842/4103. Blots were also analyzed with a probe binding *ptmG* with the same affinity in both strains (CSO-1666). 5S rRNA: loading control (CSO-0192). Magenta arrows: Processed sRNAs. Asterisks: precursor sRNAs. (b) Mapping of the 5' end of mature CJnc190 in *C. coli*. Total RNA from *C. coli* NCTC12668 wild-type and Δ180/190 was analyzed by primer extension with a probe binding the mature sRNA (CSO-4103). Lanes 3–6: Sequencing ladder for the CJnc180/190 region generated with the same primer. (c) Predicted secondary structure of *C. coli* CJnc190 and predicted interaction with *C. coli ptmG* mRNA. The secondary structure is based on its mapped 5' end and apparent northern blot length (a,b) and was predicted with RNAfold at the Vienna webserver (Lorenz et al., 2011). Bold: C/U residues of loop 2. Grey highlighting: residues identical between CJnc190 and RepG homologs. Interaction with *ptmG* mRNA was predicted with IntaRNA (Mann et al., 2017). Blue: G residues of *ptmG*. Grey C/U nucleotides of loop 2 shared with CJnc190 and RepG (1b,c). Underlined: RBS and start codon

regions revealed strong conservation of the −10 box sequence of P2, but not P1 (Figure 5b and Figure S5). Moreover, northern blot analysis detected a ~140-nt long putative precursor species in *C. coli* NCTC12668 (Figure 4a). Promoter P1 was first annotated in *C. jejuni* based on differential RNA-seq coverage, while P2 was later identified experimentally (Dugar et al., 2013; Svensson & Sharma, 2021), despite its overall stronger conservation. We also noted strong conservation of nucleotides upstream of the −10 box, which might represent a −35/−16 promoter motifs (Dugar et al., 2013; Wösten et al., 1998) or a transcription factor binding site.

To determine if CJnc190 processing might also be conserved in *C. coli*, we first performed CMfinder analysis of putative CJnc190 precursors, based on conserved promoter P2, from *C. jejuni*, *C. coli*, and *C. hepaticus* to determine if the duplex character flanking a mature (SL1-SL2) sRNA is conserved. Sequences were selected based on the conserved P2 promoter, alignment with the precursor 3' end detected in NCTC11168 (Figure S5), and consistency with the northern blot-detected length in *C. coli* of ~140 nt. The base-pairing between the two regions flanking the mature sRNA was strongly conserved among the analyzed precursors (red/colored residues, nt 72–92 and 165–185 based on NCTC11168) (Figure 5c). We were not able to delete the gene encoding RNase III in *C. coli* after repeated attempts, suggesting it is essential under routine laboratory conditions (data not shown). To obtain evidence for RNase III-mediated processing of the *C. coli* homolog in vivo, we transferred a genomic region encoding CJnc190 and 400 bp upstream of its mature 5' end into the unrelated *rdxA* locus of *C. jejuni* NCTC11168 Δ180/190. While the expression appeared to be lower in *C. jejuni* compared to *C. coli*, we were able to detect a putative mature CJnc190 sRNA of similar size as in WT (Figure S6). When the Δ*rnc* mutation was added to this strain, we no longer detected mature *C. coli* CJnc190. The 140 nt species was not detected in *C. jejuni*, even in Δ*rnc*. However, *C. jejuni* CJnc190 precursors are not stable and do not accumulate to levels of the mature sRNA (Svensson & Sharma, 2021). A similar reduction in stability could underlie the absence of detectable *C. coli*. To sum up, our observations suggest that the processing of CJnc190 precursors by RNase III is a conserved feature, even outside of *C. jejuni*.

2.7 | The CJnc180 antisense RNA appears to be a 3'UTR-derived sRNA in *C. coli*

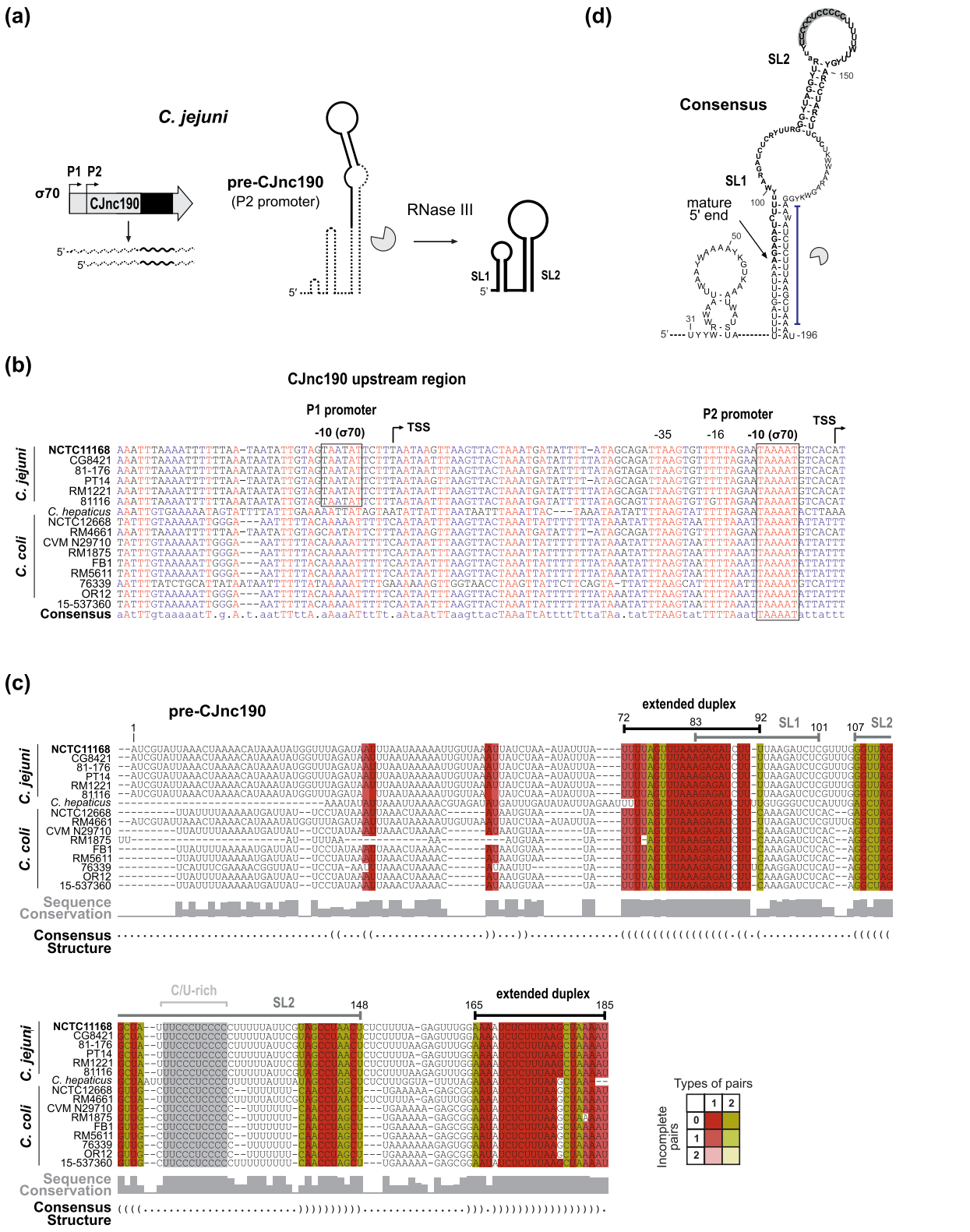
Our investigation of these sRNAs in *C. jejuni* NCTC11168 showed that while CJnc190 is processed independently of CJnc180 by RNase III via a long duplex flanking the matured sRNA, CJnc190 is required for the processing of its antisense RNA partner CJnc180 (Svensson & Sharma, 2021). Moreover, CJnc180 can antagonize the regulation of *ptmG* by CJnc190. Above, we detected a single CJnc180 species in *C. coli* NCTC12668 (Figure 4a), suggesting a possible conserved function for the asRNA outside of *C. jejuni*. However, the CJnc180 promoter is absent in most *C. coli* strains (Figure 6a and Figure S5), making it unclear how the asRNA is transcribed in *C. coli*. Primer extension mapped the CJnc180 5' end in *C. coli* NCTC12668 to a C residue upstream of the CCO0135 (encoding a FliS co-chaperone dependent on RpoN for its transcription) stop codon (Figure 6b). We did not find a sequence resembling the *C. jejuni* RpoD (TATAAT), RpoN (TTGCTT), or FliA (CGAT) sigma factor motifs (Dugar et al., 2013) immediately upstream of this position (Figure 6a). We also generated a transcriptional sfGFP (superfolder GFP) fusion to the 10th base downstream of the detected CJnc180 5' end, including ~200 bp upstream. Unlike a fusion to a similar region in *C. jejuni* that includes the CJnc180 promoter and 200 bp upstream (TSS +10 bases downstream), this construct did not produce detectable GFP fluorescence above the wild-type background when introduced into the *rdxA* locus of *C. jejuni* (Figure 6c). Thus, our data suggest that in *C. jejuni* NCTC11168, a dedicated intergenic promoter transcribes CJnc180 precursors, which are processed by RNase III in a CJnc190-dependent manner (Figure 6d). In contrast, *C. coli* NCTC12668 CJnc180 might be generated from the 3'UTR of the upstream gene or from an unidentified transcript, transcribed internally in Cj1650, via processing by a yet unidentified RNase. Overall, CJnc180 in *C. jejuni* and *C. coli* appear to be generated by markedly different biogenesis pathways and/or could represent different stages of evolution of the CJnc180 sRNA.

3 | DISCUSSION

Deep sequencing approaches have revealed base-pairing regulators encoded in diverse genomic contexts. However, few of these sRNAs have been compared between isolates or species to gain insight into how and/or why they evolved. Here, we identified CJnc190 homologs in *C. jejuni* and the related species *C. coli* and *C. hepaticus*, and found both conservation (transcription as a precursor targeted by RNase III) and diversity (promoter structure) in CJnc190 expression. We also validated the expression of the *cis*-encoded CJnc190 antagonist, CJnc180, in a *C. coli* isolate. However, unlike *C. jejuni* CJnc180, the *C. coli* sRNA overlaps with the 3'UTR of the upstream gene (CCO0135) despite having a conserved, syntenic physical location in both genomes. The intergenic promoter of *C. jejuni* CJnc180 is also not conserved in *C. coli* (Figure 6a), suggesting that the *C. coli* asRNA might be transcribed along with the upstream CCO0135 mRNA or from a promoter internal in this ORF. Although the CJnc190 target *ptmG* is sporadically conserved and horizontally exchanged within *C. jejuni* and *C. coli*, regulation by CJnc190 is maintained via conservation of its G-rich RBS. We also found that *Campylobacter* CJnc190 and RepG from *Helicobacter* share a C/U-rich loop that is crucial for targeting G-rich mRNAs of their endogenous hosts. We showed that *H. pylori* RepG can repress the CJnc190 target *ptmG* via its G-rich RBS, confirming that the sRNAs are functionally similar sRNAs, despite their strikingly different biogenesis pathways, overall limited sequence homology, and uncertain ancestries. Overall, our cross-species comparison of CJnc180/190 provides insights into how sRNAs might arise, evolve, and decay.

By exploring the biogenesis of sRNA homologs in diverse Epsilonproteobacteria, we have demonstrated that different pathways can generate a similar sRNA activity. CJnc190 precursors are transcribed from two promoters in *C. jejuni*, which could allow for environmental regulation based on the lifestyle of the isolate. Conserved Gammaproteobacterial sRNAs can also show distinct patterns of expression, such as GcvB in *Salmonella* versus *Vibrio cholerae*, whose levels are regulated inversely based on growth phase (Papenfort et al., 2015; Sharma et al., 2007). More complex pathways presumably provide additional regulatory inputs. So far, the signals and regulators that control the transcription of either RepG or CJnc190 are not known. RepG is encoded adjacent to the HP1043 orphan response regulator, which might regulate its transcription, similar to several enterobacterial sRNAs, such as SgrS, GcvA, and OxyS, which are encoded next to their transcriptional regulators (Altuvia et al., 1997; Urbanowski et al., 2000; Vanderpool & Gottesman, 2007). CJnc190 is not encoded adjacent to regulatory genes in any of the species or isolates it was detected in. While RepG transcripts are not further processed in *H. pylori*, RNase III-mediated intramolecular processing appears to be a feature of CJnc190 even outside of *C. jejuni*, based on apparent conservation of the long duplex that is required for processing in *C. jejuni*. The physiological relevance of RNase III-mediated processing of CJnc190, and when it might be rate-limiting in the biogenesis pathway, is so far unclear – although processing does increase its stability (Svensson & Sharma, 2021). Also in enterobacteria, several stand-alone sRNAs require processing for stabilization or activation (Davis & Waldor, 2007). For example, RprA and ArcZ sRNAs are expressed as precursors and require RNase E cleavage to generate functional/stable sRNAs (Chao et al., 2017). It remains to be determined if some RepG homologs outside of *H. pylori* might also be processed, or conversely, if some CJnc190 species are expressed as mature primary transcripts.

Most reported antisense transcripts are poorly conserved (Raghavan et al., 2012). Here, we also detected the antisense RNA CJnc180 in *C. coli*, suggesting that antagonism of CJnc190 that we have observed in *C. jejuni* strain NCTC11168 (Svensson & Sharma, 2021) is probably conserved. CJnc180 could also be a *trans*-acting regulator of mRNAs or even other sRNAs. Bacterial



3'UTRs are rich sources of base-pairing sRNAs, and appear to provide opportunities for network integration and autoregulation (Hoyos et al., 2020; Miller et al., 2016; Miyakoshi et al., 2015). Primer extension suggests that the *C. coli* CJnc180 5' end is generated by processing from the upstream mRNA as described for several 3'UTR-derived sRNAs, including CpxQ of *Salmonella*. It is also possible that this *C. coli* asRNA could have its own independent promoter within Cj1650 as described for *E. coli* MicL or *Salmonella* DapZ (Chao et al., 2012; Guo et al., 2014; Updegrove et al., 2019). Whether CJnc190 (or RNase III), or another RNase such as RNase J, is required for CJnc180 processing in *C. coli* remains to be seen. CJnc180 might act as a type of "excludon" where an extended antisense transcript post-transcriptionally controls the expression of an adjacent operon (Sesto et al., 2013;

FIGURE 5 Conservation of CJnc190 promoters and precursor duplex structure. (a) In *C. jejuni* NCTC11168, CJnc190 is transcribed as precursors from two promoters, P1 and P2. RNase III-dependent, but CJnc180-independent, processing of pre-CJnc190 precursors is mediated by a double-stranded region involving both ends of precursors that flank the mature sRNA (Svensson & Sharma, 2021). Processing is shown based on a single promoter (P2) precursor. (b) The CJnc190 P2, but not P1, promoter motif is highly conserved in *C. jejuni*, *C. coli*, and *C. hepaticus*. Bent arrow: TSS. Full alignment: Figure S4. (c) Structure-annotated sequence alignment of pre-CJnc190 from *Campylobacter* using CMFinder (Yao et al., 2006). Pre-CJnc190 sequences are based on the P2 TSS and the 3'-end detected in strain NCTC11168 (Svensson & Sharma, 2021). Colors indicate the number of types of pairs observed (i.e., red – one type of base-pair only). Depth of color indicates the number of incompatible pairs observed. Sequence conservation is indicated in grey below the alignment. The consensus structure is displayed using dot-bracket notation. SL1/2: stem-loops in mature CJnc190. Extended duplex: two regions (nt 72–92 and 165–185, based on NCTC11168) showing high conservation in predicted base-pairing in pre-CJnc190 species. Light grey: residues of SL2 that are identical between homologs. (d) Pre-CJnc190 consensus structure. Y – C/U; R – A/G; S – C/G; K – G/U; W – A/T. Bold residues: mature CJnc190 sRNA (based on strain NCTC11168). Grey shading: residues of loop 2 identical between homologs. Dashed line: omitted unstructured nucleotides

Toledo-Arana & Lasa, 2020). It is not yet clear if the ancestral CJnc180 was a stand-alone or 3'UTR-derived sRNA, or if CJnc190 homologs without an antisense partner exist.

It is interesting to speculate how CJnc180/190 might have arisen. Several mechanisms have been demonstrated to generate sRNAs, including de novo emergence, horizontal gene transfer, gene duplication, and exaptation/co-option of transcripts with existing functions (Dutcher & Raghavan, 2018; Jose et al., 2019; Updegrove et al., 2015). RNase III family enzymes, such as Drosha and Dicer, play a central role in the biogenesis of non-coding sRNAs such as microRNAs and siRNAs in eukaryotes (Bernstein et al., 2001; Lee et al., 2003). How the long RNase III substrate duplex of CJnc190 might have arisen is unclear. Selfish genetic elements are well known to shape bacterial genomes, and in *Coxiella burnetii*, MITEs (miniature inverted-repeat transposable elements) have been shown to generate sRNAs with long hairpins (Wachter et al., 2018).

CJnc190 and RepG are functionally similar and use C/U-rich loops to target G-rich sequences in their target mRNAs, but it is unclear if they are evolutionarily related. Detection of sRNA homologs based on sequence remains challenging (Lindgreen et al., 2014). In addition, potential examples of convergent evolution of bacterial sRNAs regulating similar pathways or even the same targets in diverse species also exist. For instance, RyhB from enterobacteria and PrrF1/2 of pseudomonads share little homology, but both regulate expression of iron-related genes (Gottesman & Storz, 2011; Massé & Gottesman, 2002; Wilderman et al., 2004). The CsrB/C sRNA antagonists of the CsrA translational regulator have evolved different numbers of GGA motifs that mediate CsrA binding, without much evidence of a shared evolutionary history. Convergent evolution also appears to have produced functionally homologous sRNAs regulating outer membrane proteins in Gammaproteobacteria: both *E. coli* MicA and *V. cholerae* VrrA regulate OmpA (Song et al., 2008; Udekwu et al., 2005), although it cannot be ruled out that they shared a common ancestor (Dutcher & Raghavan, 2018). In *Salmonella*, two sRNAs (GcvB and DapZ) have evolved to use a similar seed to target shared mRNA targets such as *oppA* and *dppA* (Chao et al., 2012). GcvB and DapZ have markedly different biogenesis pathways: GcvB is a stand-alone sRNA (Sharma et al., 2007), while DapZ is processed from the 3'UTR of the *dapB* mRNA. This potentially allows markedly different expression patterns for the two analogous sRNAs. Even CJnc180, which we detected in both *C. jejuni* and *C. coli*, might have arisen twice by convergent evolution. It is possible that small genomic changes could have led to the separate evolution of a *cis*-encoded CJnc190 antagonist with markedly different biogenesis pathways. A deeper analysis of these sRNAs between *Helicobacter* and *Campylobacter*, or between *Campylobacter* species and isolates, might reveal the evolutionary history and potential relatedness of these sRNAs.

H. pylori and *C. jejuni* rely heavily on HGT (Sheppard et al., 2018). Our observations suggest that CJnc190 in *C. coli* RM4661 might have been obtained via HGT from *C. jejuni*. RM4661 is part of *C. coli* clade 1 (Zautner et al., 2015), which shows a high level of introgression from *C. jejuni*. Over 20% of the genome of Clade 1 strains has been replaced with *C. jejuni* sequences, which might underlie the adaptation of strains from this group to agricultural niches (Sheppard et al., 2008, 2013). Bacterial sRNAs are well known to be transferred as components of mobile genetic elements and genomic islands (Padalon-Brauch et al., 2008; Pichon & Felden, 2005; Tree et al., 2014), and can even evolve to incorporate core genes into their regulons (Pfeiffer et al., 2007), including not only mRNAs but also sRNAs (Tree et al., 2014). This can impact virulence, as exemplified by horizontally-acquired *Salmonella*-specific PinT, which represses key pathogenicity island 1 (SPI-1) effectors (Barquist et al., 2016). Our observations suggest that core genome sRNAs can also recombine into new isolates, thereby replacing the sRNA allele of the host and potentially providing new regulatory opportunities. To our knowledge, the co-evolution of sRNA alleles and their targets has not yet been described. CJnc190 might, therefore, serve as a model for how sRNAs acquire horizontally transferred targets. *C. jejuni* subsp. *doylei*, which has lost a significant subset of *C. jejuni* genes (Parker et al., 2007), harbors only remnants of CJnc180/190, showing that the sRNAs have also become a casualty of genome decay. Global analysis of the conservation, function, and expression of genes encoding G-rich sequences might reveal why *C. jejuni* subsp. *doylei* has lost CJnc180/190. Moreover, it is possible that the remaining CJnc180 promoter sequence might drive transcription of a novel RNA. However, if CJnc180/190 has changed relatively recently, it is unlikely that this transcript yet has a function or is under selective pressure. Nonetheless, this locus might serve as an interesting model to

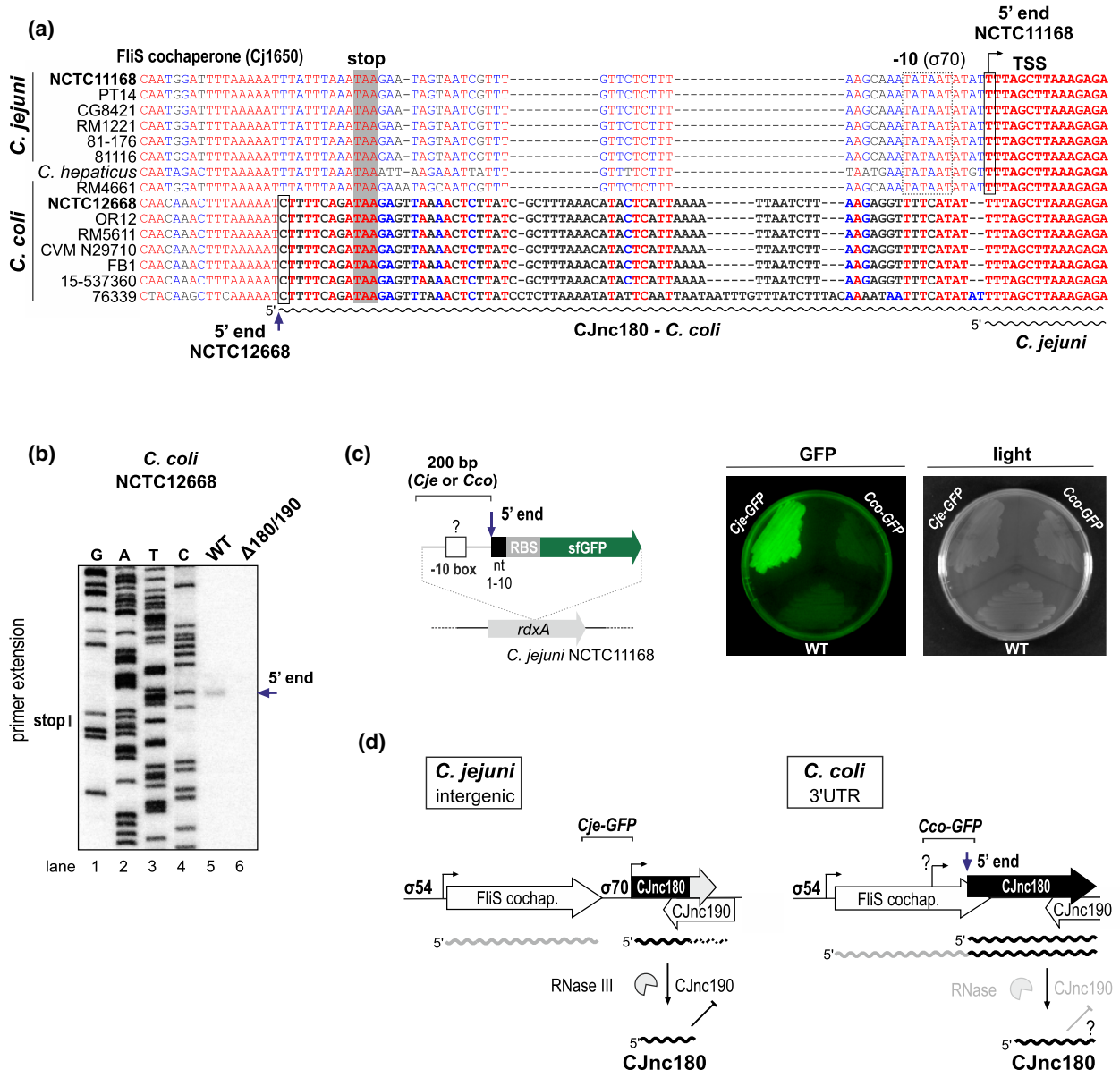


FIGURE 6 The antisense RNA CJnc180 is 3'UTR derived in *C. coli*. (a) Multiple sequence alignment of corresponding Cj1650-CJnc180 regions from different *Campylobacter* isolates. Grey: annotated FliS co-chaperone (Cj1650/CCO0135) stop codons. Bent arrow: CJnc180 TSS in *C. jejuni* (Dugar et al., 2013). Blue arrow: detected 5' end in *C. coli* NCTC12668. CJnc180 regions in *C. jejuni* and *C. coli* are indicated underneath the alignment. (b) Primer extension-based mapping of the *C. coli* CJnc180 5' end. Total RNA from *C. coli* NCTC12668 wild-type or a Δ180/190 mutant was annealed with a radiolabeled probe specific for the complement of the CJnc190 loop (CSO-3842) and extended with reverse transcriptase. Lanes 1–4: sequencing ladder generated with the same primer. The stop codon of the upstream gene (CCO0135) is indicated on the left. (c) GFP reporter expression for transcriptional fusions to regions upstream of detected CJnc180 5' ends from *C. jejuni* NCTC11168 and *C. coli* NCTC12668. *Left*: ~200 bp upstream and 10 bp downstream of the mapped 5' end of CJnc180 from *C. jejuni* (*Cje*) or *C. coli* (*Cco*) was fused to the *hupB* RBS and sfGFP. The construct was introduced into *rdxA* of *C. jejuni* NCTC11168 (see also panel d). *Right*: GFP fluorescence or growth on MH agar for the *C. jejuni* and *C. coli* CJnc180 transcriptional fusions versus the *C. jejuni* wild-type strain. (d) CJnc180 biogenesis in *C. jejuni* versus *C. coli*. In *C. jejuni*, a dedicated promoter drives the expression of a CJnc180 precursor, which is processed by RNase III together with CJnc190, into the mature sRNA. In *C. coli*, CJnc180 appears to be co-transcribed with and released from the upstream RpoN(σ^{54})-dependent mRNA by a yet unidentified RNase, or might also be transcribed from an internal promoter and also processed

explore the generation of new sRNA species from genome rearrangements, as has been reported in *E. coli* (Raghavan et al., 2015).

CJnc190 likely has additional targets related to infection (Alzheimer et al., 2020), and our in silico predictions suggest CJnc190 might target several G-rich mRNAs of the flagellin modification or capsular polysaccharide-related islands. While these targets require validation, they are strong candidates for a role in virulence, as such surface structures are well-described players

in *C. jejuni* host interactions (Burnham & Hendrixson, 2018). Like *H. pylori*, *C. jejuni* extensively uses phase-variation at simple sequence repeats such as homopolymeric G-tracts to generate diversity (Bayliss et al., 2012). Length variation of such G-tracts generates in-frame stop codons, preventing translation of the full-length protein in so-called contingency loci. Several putative CJnc190 targets were predicted to be bound via phase-variable homopolymeric G-tracts in ORFs. If CJnc190 can in fact base-pair with such sequences, it is not clear what the consequences of this would be. CJnc190 and RepG are part of a class of bacterial sRNAs that use C/U-rich apical loops to target G-rich sequences in mRNAs, which might use similar targeting rules. Such sRNAs have been identified in pathogens such as *Xanthomonas campestris*, *Staphylococcus aureus*, *Listeria monocytogenes*, *Neisseria meningitidis*, and *Mycobacterium tuberculosis*, suggesting they might represent a novel class of virulence regulators (Bronesky et al., 2016; Heidrich et al., 2017; Mai et al., 2019; Mollerup et al., 2016; Pannekoek et al., 2017; Schmidtke et al., 2013; Sievers et al., 2015). However, the targeting of longer phase-variable/G-rich tracts by RepG and CJnc190, together with their extended C/U-rich motif compared to other sRNAs, set them apart from most other examples. A curious set of paralogous sRNAs identified in *Rhodobacter sphaeroides*, each of which has a CCUCCUCCC motif in adjacent stem-loops, appear to define a similarly unique family of sRNAs conserved in Alphaproteobacteria, although their targeting mechanism has not yet been demonstrated (Berghoff et al., 2009; Billenkamp et al., 2015; Reinkensmeier & Giegerich, 2015). Several pathogens rely on simple sequence repeats to generate reversible genetic diversity related to virulence. Some of these could also encode RepG and/or CJnc190-like sRNAs. Uncovering the so far unknown signals controlling transcription, processing, and/or turnover of CJnc190 (and RepG), as well as identification of their targetomes, will provide insight into their physiological role in the cell and how they influence pathogenesis. In addition, examination of their regulation and physiological role in a comparative manner between isolates will reveal how sRNAs and their regulons might evolve.

4 | MATERIALS AND METHODS

4.1 | Bacterial strains and culture conditions

All *Campylobacter* strains (Table S2) were routinely grown either on Müller-Hinton (MH) agar plates or with shaking at 140 rpm in *Brucella* broth (BB) at 37°C in a microaerobic atmosphere (10% CO_2, 5% O_2). All *Campylobacter* media was supplemented with 10 μg/ml vancomycin. Agar was supplemented with marker-selective antibiotics [20 μg/ml chloramphenicol (Cm), 50 μg/ml kanamycin (Kan), or 20 μg/ml gentamicin (Gm)], where appropriate. *E. coli* strains were grown aerobically at 37°C in lysogeny broth (LB) or on LB agar supplemented with the appropriate antibiotics for marker selection.

4.2 | General recombinant DNA techniques

Oligonucleotide primers for PCR, northern blot detection, and reverse transcription are listed in Table S3. Oligonucleotides were purchased from Sigma. DNA constructs and mutations were confirmed by Sanger sequencing (Macrogen). Restriction enzymes, *Taq* polymerase for validation PCR, and T4 DNA ligase were purchased from NEB. For cloning purposes, Phusion high-fidelity DNA polymerase (Thermo Fisher Scientific) was used.

4.3 | Transformation of *C. jejuni* and *C. coli* for mutant construction

All *Campylobacter* mutant strains constructed for this work (deletion, complementation at *rdxA*) were constructed by double-crossover homologous recombination with DNA fragments introduced by electroporation as follows. Strains grown from frozen stocks until passage one or two on MH agar were harvested into cold electroporation solution (272 mM sucrose, 15% (v/v) glycerol) and washed twice with the same solution. Cells (50 μl) were mixed with 200–400 ng PCR product on the ice and electroporated (Bio-rad MicroPulser) in a 1mm gap cuvette at 2.5 kV. Cells were then transferred with *Brucella* broth to a non-selective MH plate and recovered overnight at 37°C microaerobically before plating on the appropriate selective medium.

4.4 | Generation of CJnc180/190 mutant strains in *C. jejuni* and *C. coli*

The putative CJnc180/190 region from *C. coli* strain NCTC12668 was deleted via homologous recombination with a Kan^R cassette. Briefly, the sequence of the CJnc180/190 region of strain NCTC12668 (CSS-0934) was first determined by Sanger sequencing with primers CSO-3953 and CSO-3954. Next, approximately 500 bp upstream and downstream of the region to be deleted was amplified using CSO-3953/4002 and CSO-4003/3955 from *C. coli* WT genomic DNA (CSS-0934), respectively, to also introduced regions overlapping a non-polar Kan^R cassette. The upstream and downstream regions were then mixed in approximately equimolar ratios with the resistance cassette (amplified with HPK1/HPK2), annealed and extended, and then amplified by PCR with primers CSO-3953/3955. The resulting amplicon was electroporated into *C. coli* NCTC12668 WT, and Kan^R colonies were validated for deletion of CJnc180/190 and insertion of the resistance cassette by colony PCR using CSO-4024/HPK2.

To express the CJnc180/190 homologous region from *C. coli* NCTC12668 in *C. jejuni* NCTC11168, we used a similar approach as used previously to generate the *C. jejuni* NCTC11168 C-180/190 complemented strain (Alzheimer et al., 2020). The CJnc180/190 region (including 200 bp upstream of the CJnc180 5' end and 400 bp upstream of the CJnc190 mature 5' end) was amplified from genomic DNA from *C. coli* NCTC12668 WT (CSS-0934) using primers CSO-4533/4534. Plasmid pGD34.7 (Alzheimer et al., 2020) was

amplified with CSO-0347/0350. Vector and insert were digested with *Nde*I/*Cla*I and ligated together, and the resulting plasmid (pSSv132.1) was validated by colony PCR (CSO-0643/3270) and sequencing with CSO-0643. The insert containing *rdxA* flanking a Cm^R cassette and *C. coli* CJnc180/190 was amplified using CSO-2276/2277 and electroporated into *C. jejuni* NCTC11168 Δ180/190 (CSS-1157). Insertion of the construct at *rdxA* was validated by colony PCR (CSO-0643/0349) and sequencing (CSO-0643). The *rnc* deletion was added by electroporation of a PCR product (CSO-0242/0243) from CSS-2438. The resulting strain was validated for *rnc* deletion with a non-polar Hyg^R cassette by colony PCR with CSO-0240/2857.

4.5 | Heterologous expression of *H. pylori* RepG from the *rdxA* locus in *C. jejuni*

A construct for *H. pylori* RepG expression in *C. jejuni* NCTC11168 by insertion at the neutral *rdxA* locus (Ribardo et al., 2010) was generated by ligation of *repG* from strain 26695, including its native promoter (amplified with CSO-0424/0426 from CSS-0004), into a plasmid containing ~500 bp of upstream and downstream sequence from *C.jejuni rdxA* flanking a Cm^R cassette (with promoter and terminator) (Alzheimer et al., 2020). The cassette for electroporation into *C. jejuni* strain NCTC11168 Δ180/190 (CSS-1157) was amplified from this plasmid (pSSv7.1) with CSO-2276/2277. Insertion of the construct at *rdxA* was validated by colony PCR (CSO-0643/0349) and sequencing (CSO-0643).

4.6 | Total protein analysis by SDS-PAGE and western blotting

Analyses of protein expression in *C. jejuni* were performed by SDS-PAGE and western blotting. Bacterial cells were collected from cultures in the mid-log phase (OD_{600} 0.4–0.5) by centrifugation at 11,000*g* for 3 min. Cell pellets were resuspended in 100 μl of 1× protein loading buffer (62.5 mM Tris-HCl, pH 6.8, 100 mM DTT, 10% (v/v) glycerol, 2% (w/v) SDS, 0.01% (w/v) bromophenol blue) and boiled for 8 min. For analysis of total proteins, 0.05–0.1 OD_{600} of cells were loaded per lane on 12% SDS-polyacrylamide (PAA) gels. Gels were stained with PageBlue (Thermo Fisher Scientific). For western blot analysis, samples corresponding to an OD_{600} of 0.05–0.1 were separated on 12% SDS-PAA gels and transferred to a nitrocellulose membrane by semi-dry blotting. Membranes were blocked for 1 hr with 10% (w/v) milk powder in TBS-T (Tris-buffered saline-Tween-20) and then incubated overnight with primary antibody (monoclonal anti-FLAG, 1:1,000 in 3% BSA/TBS-T; Sigma-Aldrich, #F1804-1MG) at 4°C. Membranes were then washed with TBS-T, followed by 1 hr incubation with secondary antibody (anti-mouse IgG, 1:10,000 in 3% BSA/TBS-T; GE Healthcare, #RPN4201). After washing, the blot was developed using an enhanced chemiluminescence reagent. A monoclonal antibody specific for GroEL (1:10,000 in 3% BSA/TBS-T; Sigma-Aldrich, # G6532-5Ml) with an anti-rabbit IgG (1:10,000 in 3% BSA/TBS-T; GE Healthcare, #RPN4301) secondary antibody was used as a loading control.

4.7 | Total RNA extraction and analysis by northern blotting

For the analysis of total RNA, bacterial strains were grown to log phase in BB and ~4 OD_{600} were harvested and mixed with 0.2 volumes of stop-mix (95% ethanol and 5% phenol, v/v). Samples were immediately snap-frozen in liquid nitrogen and stored at −80°C until RNA extraction. Frozen samples were thawed on ice and centrifuged at 4°C to collect cell pellets (4,500*g*, 20 min). Cell pellets were lysed by resuspension in 600 μl of a solution containing 0.5 mg/ml lysozyme in Tris-EDTA buffer (pH 8.0) and 60 μl of 10% SDS and incubated for 2 min at 64°C. Afterward, the total RNA was extracted using the hot-phenol method as described previously (Sharma et al., 2010). For northern blot analysis, 10 μg of total RNA in Gel Loading Buffer II (GLII, Ambion) was loaded per lane on 6% PAA/7 M urea denaturing gels. Following electrophoretic separation, RNA was transferred to Hybond-XL membranes (GE Healthcare) by electroblotting. Transferred RNA was then cross-linked to the membrane with ultraviolet light to the membrane and hybridized with γ^{32}P-ATP 5'-end-labeled DNA oligonucleotides (Table S3) in Roti Hybri-quick (Roth). Blots were dried, exposed to a phosphorimager screen, and then scanned (FLA-3000 Series, Fuji).

4.8 | Primer extension analysis of RNA 5' ends

To map 5' ends of transcripts present in vivo, the total RNA was extracted from bacteria grown to log phase. RNA was digested with DNase I (Thermo Fisher Scientific) to remove DNA, and then 5–10 μg of RNA was added up to 5.5 μl H_2O, denatured, and snap-cooled on ice. A radioactively labeled (5'-end) DNA oligonucleotide complementary to the RNA of interest was then added (Table S3). The mixture was heated to 80°C and then slowly cooled (1°C per min) to allow annealing of the primer. Once the mixture reached 42°C, a master mix with RT buffer and 20 U Maxima Reverse Transcriptase (Thermo Fisher Scientific) was added. The reaction was then allowed to proceed for 1 hr at 50°C. Reactions were stopped with 12 μl GLII (Ambion). A sequencing ladder was also constructed using the DNA cycle sequencing kit (Jena Bioscience) according to the manufacturer's instructions with the CJnc180/190 region amplified with primers CSO-3953/3954 (*C. coli* NCTC12668) from genomic DNA as a template and the same radioactively-labeled primer was used for the reverse transcription reaction. Reactions were separated on 6% or 10% PAA-urea sequencing gels, which were then dried and exposed to a phosphorimager screen, and then scanned (FLA-3000 Series, Fuji). The following primers were used for primer extension: CJnc190 (*C. coli*), CSO-4103; CJnc180 (*C. coli*), CSO-3842.

4.9 | In vitro transcription and 5'-end-labeling of RNAs

PCR with Phusion polymerase was used to generate DNA templates containing the T7 promoter sequence using oligonucleotides listed in Table S3 or as previously described (Pernitzsch et al., 2014; Svensson & Sharma, 2021). Briefly, templates for T7 transcription were generated using the following primer sets: RepG (87 nt): JVO-5125/5126 on CSS-0004, *tlpB*(12G)(217 nt): JVO-5127/5143 on CSS-0004; CJnc190 (70 nt): CSO-2184/1972 on CSS-5295, *ptmG* leader (204 nt starting from TSS): CSO-1665/2956 on CSS-5295, *ptmG* leader (100 nt starting from TSS): CSO-1665/1666 on CSS-5295. Transcription of RNAs in vitro by T7 RNA polymerase was then carried out using the MEGAscript T7 kit (Ambion) according to the manufacturer's instructions. RNAs were then checked for quality by electrophoresis on a PAA-urea gel, dephosphorylated with Antarctic Phosphatase (NEB), 5′-end-labelled (γ^{32}P) with polynucleotide kinase (NEB), and purified by gel extraction as previously described (Papenfort et al., 2006).

4.10 | Electrophoretic mobility shift assays (EMSAs)

Gel-shift assays were performed as described previously (Pernitzsch et al., 2014). Briefly, 5′ end radiolabeled RNA (0.04 pmol) was denatured (1 min, 95°C) and cooled for 5 min on ice. Yeast tRNA (1 μg, Ambion) and 1 μl of 10× RNA structure buffer (Ambion; 10 mM Tris, pH 7, 100 mM KCl, 10 mM $MgCl_2$) were then mixed with the labeled RNA. Unlabeled RNA (2 μl diluted in 1× Structure Buffer) was added to the desired final concentrations (0, 10, 20, 50, 100, 200, 500, or 1 μM). Binding reactions were incubated at 37°C for 15 min. Before loading on a pre-cooled native 6% PAA, 0.5× TBE gel, samples were mixed with 3 μl native loading buffer [50% (v/v) glycerol, 0.5× TBE, 0.2% (w/v) bromophenol blue]. Gels were run in 0.5× TBE buffer at 300 V and 4°C, following which they were dried, exposed to a phosphorimager screen, and scanned (FLA-3000 Series, Fuji).

4.11 | Inline probing

Inline probing assays for RNA structure and binding interactions in vitro were performed essentially as described previously (Pernitzsch et al., 2014). Five prime end-labeled RNAs (0.2 pmol, see above) in 5 μl water were mixed with an equal volume of 2× Inline buffer (100 mM Tris-HCl, pH 8.3, 40 mM $MgCl_2$, and 200 mM KCl and incubated for 40 hr at room temperature to allow spontaneous cleavage. Reactions were stopped with an equal volume of 2× colorless loading buffer (10 M urea and 1.5 mM EDTA, pH 8.0). Reactions were separated on 6% or 10% PAA-urea sequencing gels, which were dried and exposed to a PhosphorImager screen. RNA ladders were prepared using alkaline hydrolysis buffer (OH ladder) or sequencing buffer (T1 ladder) according to the manufacturer's instructions (Ambion).

4.12 | RNA–RNA interaction predictions

Genome-wide predictions were performed with IntaRNA (Mann et al., 2017) version 3.2.0 (linking Vienna RNA package 2.4.14) using default parameters, except for seed size of 6 nt and a region of 300 nt up- and downstream of the start codon. The mature CJnc180 or CJnc190 sequences from *C. jejuni* NCTC11168 and RepG sequence from *H. pylori* 26695 were used as inputs (Pernitzsch et al., 2014; Svensson & Sharma, 2021). The NCTC11168 genome accession used for predictions was NC_002163. Base-pairing between sRNAs and single targets was also predicted using IntaRNA with 200 nt upstream of the annotated start codon.

4.13 | Transcriptional fusions to superfolder GFP

The CJnc180 promoter from *C. jejuni* NCTC11168 and CJnc180 upstream region from *C. coli* NCTC12668 were fused to a promoterless superfolder GFP (sfGFP) cassette with the RBS from *hupB* (Cj0913c) by overlap PCR. First, an sfGFP-Kan^R-*rdx*AUP(~500 bp) cassette was generated in a plasmid. The sfGFP gene from pXG10 (Corcoran et al., 2012) was amplified with primers CSO-3279/3569, while pST1 (Dugar et al., 2018) was amplified with CSO-0762/0347. Insert and vector were digested with *Xma*I and ligated together. The cassette was validated by colony PCR with CSO-3270/0023 and sequenced with CSO-0023. The sfGFP-Kan^R-*rdx*AUP cassette was then amplified with primers CSO-5590/2276 to introduce the strong *hupB* RBS, and the *rdx*ADN region (~500 bp) was amplified with CSO-0347/2277. CJnc180 promoter/upstream regions were amplified with CSO-5595/5593 and CSO-5597/5598 (*C. jejuni* NCTC11168 and *C. coli* NCTC12668, respectively) from wild-type genomic DNA from the respective strains. This introduced regions overlapping the *rdx*ADN fragment or *hupB* RBS. The three fragments (*rdx*ADN/promoter/sfGFP-Kan^R-*rdx*AUP) were mixed, annealed, and amplified by overlap PCR with CSO-2276/2277. The resulting PCR product was electroporated into *C. jejuni* NCTC11168 wild-type. Kan^R colonies (*C. jejuni* fusion, CSS-7559, and *C. coli* fusion, CSS-7586) were validated for insertion of the transcriptional fusion at *rdxA* by colony PCR with CSO-0349/0789, and promoter regions were checked by sequencing with CSO-3270.

4.14 | Conservation analyses and multiple sequence alignments

Epsilonproteobacteria strains are listed in Table S4. Putative CJnc190 homologs were identified in *C. jejuni*, *C. coli* with BLAST or manual inspection of the intergenic region between Cj1650 and Cj1651c (*C. hepaticus*). For alignment of putative CJnc180/190 regions between Cj1650 and Cj1651c (*map*) homologs were retrieved from KEGG. For *C. coli* NCTC12668, the CJnc180/190, *ptmG*, and *gyrB* regions were determined by Sanger sequencing. Homologs of *ptmG* were identified via tBLASTn at NCBI using a word size of 3, and the annotated start codon and 70 nt upstream were aligned to identify conserved

G-tracts. Multiple sequence alignments and the *gyrB* phylogenetic tree were constructed at phylogeny.fr (Dereeper et al., 2008).

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