The widespread CsrA/RsmA protein regulators repress translation by binding GGA motifs in bacterial mRNAs. CsrA activity is primarily controlled through sequestration by multiple small regulatory RNAs. Here we investigate CsrA activity control in the absence of antagonizing small RNAs by examining the CsrA regulon in the human pathogen Campylobacter jejuni. We use genome-wide co-immunoprecipitation combined with RNA sequencing to show that CsrA primarily binds flagellar mRNAs and identify the major flagellin mRNA (flaA) as the main CsrA target. The flaA mRNA is translationally repressed by CsrA, but it can also titrate CsrA activity. Together with the main C. jejuni CsrA antagonist, the FliW protein, flaA mRNA controls CsrA-mediated post-transcriptional regulation of other flagellar genes. RNA-FISH reveals that flaA mRNA is expressed and localized at the poles of elongating cells. Polar flaA mRNA localization is translation dependent and is post-transcriptionally regulated by the CsrA-FliW network. Overall, our results suggest a role for CsrA-FliW in spatiotemporal control of flagella assembly and localization of a dual-function mRNA.
Bacterial RNA localization, even less is known about how this process may be regulated and which, if any, RBPs are involved. Here we show, based on a variety of C. jejuni mutants that disrupt or maintain flaA mRNA localization, that polar flaA mRNA localization requires its translation. Furthermore, we demonstrate that FliW facilitates polar flagellin mRNA localization by antagonizing CsrA-mediated translational repression of flaA. The unexpected role of the CsrA-FliW system in spatial control of flagellin mRNA expression provides new insight into the role of RBPs in bacterial mRNA localization, a process only recently described in prokaryotes.

Results

Global RIP-seq reveals direct CsrA targets in C. jejuni. To globally identify C. jejuni CsrA targets and any RNA regulators of CsrA activity, we applied a RIP-seq approach\(^\text{17,18}\). The csrA (Cj1103) gene was chromosomally 3xFLAG-tagged at its C-terminus in strains NCTC11168 and 81-176. CsrA-3xFLAG is constitutively expressed during growth in rich medium, and neither introduction of the FLAG-tag nor deletion of csrA affects C. jejuni growth under the examined conditions (Supplementary Fig. 1). We performed co-IPs on mid-maximal-phase lysates of csrA-3xFLAG strains and, as control, their respective untagged wild-type (WT) strains (Fig. 1a and Supplementary Fig. 2a). After conversion of co-purified RNAs into cDNA and deep sequencing, 93.2–95.8% of the 4.6–6.2 million sequenced reads for the individual libraries were mapped to the respective genomes (Supplementary Table 1). Most of the NCTC11168 control-coIP library reads mapped to presumably non-specifically pulled-down abundant classes of RNA (rRNA, tRNA and housekeeping RNAs; Fig. 1b and Supplementary Table 2). In contrast, a ∼36-fold and ∼5-fold enrichment for reads mapped to 5’UTRs or open reading frames (ORFs) of mRNAs, respectively, was observed in the CsrA-3xFLAG coIP library (Fig. 1b). No specific sRNA enrichment was detected. As the coIP of strain 81–176 showed similar enrichment patterns (Supplementary Fig. 2b), we focused on strain NCTC11168.

C. jejuni CsrA primarily binds flagellar mRNAs. Functional enrichment analysis of the 154 top CsrA targets with >5-fold enrichment in the CsrA-3xFLAG- versus control-coIP (Supplementary Data 1) revealed an overrepresentation of mRNAs from the class ‘Surface Structures’, including flagellar genes (Supplementary Fig. 3a,b). In fact, 90% of the reads mapping to the >5-fold-enriched CsrA targets belonged to flagella- or motility-related genes (Fig. 1c). The alternative sigma factors RpoN (\(\sigma^\text{s}\)) and FliA (\(\sigma^\text{FL}\)) hierarchically control flagellar expression in Campylobacter\(^\text{22}\). Early genes are expressed from RpoD/\(\sigma^\text{70}\)-dependent promoters, whereas class 2 (middle) and class 3 (late) genes are RpoN- and FliA-dependent, respectively\(^\text{22}\).

Most of the enriched transcripts belonged to either class 2 or class 3 (Table 1 and Supplementary Fig. 3c). The most abundantly co-purified transcript, with more than 300-fold enrichment, was flaA mRNA, encoding the major flagellin (Fig. 1c). cDNA peaks reveal CsrA binds in diverse mRNA regions. Visual inspection of the cDNA read-patterns showed that numerous flagellar mRNAs, including flaA, flaG and flgI (encoding the major flagellin, a gene involved in flagellum formation, and a P-ring component, respectively) showed strong enrichment in their 5’UTRs (Fig. 1d and Supplementary Fig. 4a).

CsrA binding was also observed between two genes in poly-cistronic mRNAs, such as the Cj0310c-Cj0309c and Cj0805-dapA operons. Analysis of the potential CsrA-binding sites in an Escherichia coli green fluorescent protein (GFP) reporter-system,

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**Post-transcriptional control involves a complex interplay between mRNAs, small regulatory RNAs (sRNAs) and protein regulators. Although regulatory functions have typically been attributed to proteins or sRNAs, mRNAs have canonically been considered as targets of this regulation. However, regulatory functions have recently also been described for mRNAs that either encode sRNAs in their untranslated regions (UTRs) or act as sponges that sequester other regulatory factors\(^\text{1–4}\). The widespread bacterial Csr/Rsm (Carbon storage regulator/Regulator of secondary metabolism) regulatory network\(^\text{2}\) is an ideal model system to study the complex post-transcriptional cross-talk between mRNAs, sRNAs and protein regulators. About 75% of all sequenced bacterial genomes encode a homologue of the central RNA-binding protein (RBP) of this system, CsrA (RsmA/E). CsrA is a pleiotropic regulator of global physiological processes\(^\text{3}\) and considered the most conserved post-transcriptional virulence regulator\(^\text{6}\). CsrA mainly acts by repression of translation initiation via binding to GGA-rich motifs that are often located in hairpin loops and/or overlap the Shine-Dalgarno (SD) sequence\(^\text{5}\). In Gammaproteobacteria, CsrA activity is regulated through the CsrB/C and RsmX/Y/Z families of sRNAs\(^\text{5,7}\). These antagonizing sRNAs are often induced by environmental signals\(^\text{6}\) and harbour multiple stem-loops with high-affinity GGA motifs that sequester CsrA/RsmA\(^\text{8}\). Despite the presence of CsrA, many bacteria lack homologues of these antagonizing sRNAs. Also, the global CsrA regulon and its general biological function outside the Gammaproteobacteria are unclear. In the Gram-positive \textit{Bacillus subtilis}, the flagellar assembly protein FliW antagonizes CsrA via direct binding\(^\text{9}\). Although FliW homologues are relatively widespread\(^\text{9}\), protein-mediated regulation of CsrA has not yet been shown outside \textit{B. subtilis}. Whether FliW can cooperate with RNA-mediated regulation of CsrA is also unknown.

In the Gram-negative Epsilonproteobacterium \textit{Campylobacter jejuni}, currently the leading cause of bacterial gastroenteritis in humans, CsrA affects motility, biofilm formation, oxidative stress response and infection\(^\text{10}\). Despite several phenotypic analyses of \textit{csrA} deletion strains\(^\text{10–12}\), direct CsrA targets in Epsilonproteobacteria are largely unknown. Global transcriptome studies indicated that both \textit{C. jejuni} and the related pathogen \textit{Helicobacter pylori}\(^\text{13–16}\), which both carry potential FliW homologues, lack the CsrA-antagonizing sRNAs.

Here we use co-immunoprecipitation (co-IP) combined with RNA sequencing\(^\text{17,18}\) (RIP-seq) to globally determine the direct RNA-binding partners of \textit{C. jejuni} and investigate whether RNA-based regulation of CsrA occurs in the absence of canonical antagonizing sRNAs. Our genome-wide approach reveals many mRNAs of flagellar genes as potential CsrA targets and we demonstrate that \textit{flaA} mRNA, encoding the major flagellin, has dual (coding and regulatory) function. As the most abundantly co-purified transcript, \textit{flaA} mRNA is the main target of CsrA translational repression. In addition, the \textit{flaA} leader can act as an mRNA-derived RNA antagonist of CsrA. Together with the main CsrA antagonist, the FliW protein, \textit{flaA} mRNA titrates CsrA to regulate expression of other flagellar genes.

In addition, using confocal and super-resolution microscopy imaging, we show that \textit{flaA} mRNA is expressed in elongating cells and localizes to the cell poles of the amphitrichous \textit{C. jejuni}. In contrast to eukaryotes\(^\text{19}\), RNA localization is so far only poorly understood in prokaryotes. Bacterial mRNAs can remain localized close to their genomic site of transcription\(^\text{20}\) or can migrate to places in the cell where their encoded products are required in a translation-independent manner involving cis-acting signals in the RNA itself\(^\text{21}\). Besides the mechanisms of
originally developed to study sRNA-mediated regulation\textsuperscript{23}, revealed all of the tested 5′ UTR targets (flaA, flaG, flgI, flgH, flgE, pseB and Cj1249) were highly upregulated (>10-fold) in the absence of E. coli csrA as measured by western blot and FACS analyses (Fig. 1d and Supplementary Figs 4 and 5). Reduced reporter fusion expression was restored by complementation of ΔcsrA with C. jejuni csrA. Using an operon reporter, where the C-terminal part of the upstream gene is fused to FLAG-GFP or a FLAG-3xFLAG tag (Supplementary Fig. 4b), as deletion of csrA dramatically enhanced biofilm formation and led to poor growth in liquid culture in our strain, reporter experiments were performed in a ΔcsrA backgrounds. GroEL served as loading control. Protein samples corresponding to 0.1 OD\textsubscript{600} were loaded. Quantifications of reporter expression are given below the blots. (e) (Left) CsrA-binding motif predicted by MEME\textsuperscript{24} (E-value = 2.1E-11). (Right) Consensus secondary structure motif of C. jejuni CsrA-binding sites predicted by CMfinder\textsuperscript{62}.

Automated peak-detection reveals a CsrA-binding motif. To automatically identify CsrA-binding regions and a binding motif from coIP cDNA enrichment patterns, we developed a peak-detection algorithm based on a sliding window approach (see the Methods for details). This approach predicted 328 potential CsrA-binding sites with >5-fold enrichment in the NCTC11168 coIP (Supplementary Data 2). As a control, peak

Figure 1 | RIP-seq analysis of C. jejuni CsrA. (a) Western blot analysis of coIP samples of C. jejuni NCTC11168 WT and csrA-3xFLAG strains using anti-FLAG antibody confirms a successful CsrA-3xFLAG pulldown in the tagged strain. The amount of samples loaded (OD\textsubscript{600} of bacteria) is indicated. GroEL served as loading control. (b) Pie charts showing relative proportions of mapped cDNA reads of different RNA classes in the coIP libraries (hsRNA: housekeeping RNAs). Numbers in brackets indicate the relative enrichment of the respective RNA class in the CsrA-3xFLAG versus control coIPs. (c) Pie chart showing the percentages and enriched genes of mapped reads for all >5-fold enriched CsrA target genes. (d) (Left) Mapped RNA-seq reads for the control (black) and CsrA-3xFLAG coIP (blue) in strain NCTC11168. Grey arrows: ORFs; black arrows: transcriptional start sites (TSS). Examples of enrichment patterns in 5′ UTRs (flaA) and between genes in a polycistron (Cj0310c-Cj0309c operon; encoding two paralogous efflux proteins). (Right) Western blot analysis using anti-FLAG and anti-GFP antibodies of reporter fusions to potential C. jejuni CsrA target genes in E. coli ΔpgaA, ΔpgaA/ΔcsrA and ΔpgaA/ΔcsrA/pBAD-csrA\textsubscript{Cj} (complementation with C. jejuni CsrA-3xFLAG-Cj0310c operon encoding two paralogous efflux proteins).
Table 1 | Enrichment of genes involved in flagellar biosynthesis in the CsrA coIP data.

| Enrichment (reads) | C. jejuni NCTC11168 | C. jejuni 81-176 |
|--------------------|---------------------|-------------------|
| **Regulation of expression (class 1)** | | |
| fliP (Cj0820c) | 1.6 (7) | 1.6 (181) |
| fliO (Cj0352) | – | 1.2 (299) |
| flgG (Cj0697) | 4.3 (8,133) | 77.4 (9,670) |
| flgH (Cj0698) | 1.2 (1) | 1.4 (253) |
| flgI (Cj1463) | – | 4.4 (180) |
| flgJ (Cj1462) | 170.5 (5,750) | 52.7 (12,087) |
| flgK (Cj0769c) | 0.8 (2) | 15.8 (410) |
| flgH (Cj0687c) | 200.9 (1,911) | 20.1 (3,288) |
| **Flagellar hook components (class 2)** | | |
| flgE (Cj1729c) | – | 68.2 (104,324) |
| flgD (Cj0042) | – | 3.6 (1015) |
| flgE (Cj0526c) | 1.6 (7) | 1.6 (181) |
| Cj0040* | 356.2 (3,389) | 110.6 (9,277) |
| flgG (Cj1466) | – | 0.7 (4) |
| flgI (Cj0887c) | – | 2.0 (484) |
| **Flagellar filament components (classes 2 and 3)** | | |
| fliA (Cj1339c) | 304.5 (693,471) | 111 (473,588) |
| fliB (Cj1338c) | 58.8 (915) | 141 (17,880) |
| flgJ (Cj0548) | – | 6.8 (4,348) |
| flgI (Cj0549) | – | 1.6 (149) |
| fliC (Cj0720) | 1.2 (344) | 1.2 (1,298) |
| **Other enriched genes (>5x) involved in flagella formation** | | |
| pseB (Cj1293) | 119.7 (2,298) | 9.5 (2,280) |
| pseB (Cj1317) | 1.7 (22) | 7.2 (864) |
| flgA (Cj0547) | 346.1 (11,077) | 72.4 (18,150) |
| motA (Cj0337c) | 10.3 (89) | 1.8 (660) |
| Cj0951c | 15.2 (79) | 2.3 (3) |
| Cj0248 | 5.5 (120) | 1.8 (387) |
| flhX (Cj0848c) | – | 7.5 (13) |

CoIP, co-immunoprecipitation; UTR, untranslated region; ORF, open reading frame. Classification of flagellar genes is based on ref. 7. Transcripts with >5-fold enrichment in cDNA read counts in the CsrA-3xFLAG versus control coIP libraries are highlighted in bold. Numbers in brackets indicate the absolute cDNA read counts in the CsrA-3xFLAG coIP libraries.

*Cj0040 (unknown function) is the first gene of the hook gene operon.

detection was performed in reverse manner by scanning for enriched regions in the control- versus CsrA-3xFLAG-coIP. This analysis revealed only five peaks, without a common motif, indicating a high specificity of the peaks detected in the CsrA-3xFLAG-coIP. MEME 24 analysis of the 328 enriched sequences revealed a (C/A)(A/T)GGA motif in 324/328 input sequences (Fig. 1e). Analysis of the 81-176 coIP led to a similar motif (Supplementary Fig. 2c). To check if a similar motif can be found in non-enriched regions, we conducted the peak-detection in reverse manner using a cutoff of only >1-fold enrichment in the control- versus CsrA-3xFLAG-coIP. This revealed 448 ‘enriched’ sites in the control library. Subsequent motif prediction did not yield any significant motifs, further supporting high specificity of the coIP approach. Consensus-structure motif screening of the enriched CsrA-coIP sequences revealed an AAGGA motif in a hairpin-structure loop in 276/328 input sequences (Fig. 1e). These C. jejuni sequence/structural motifs agree with binding sites of other CsrA homologues25.
flaA mRNA is translationally repressed by CsrA. The flagellar filament, consisting mainly of the FlaA flagellin, is among the last components produced during flagellum assembly. In our coIP, 77% of the reads from >5-fold enriched genes mapped to flaA, indicating it as the main CsrA target (Fig. 1c). Secondary-structure predictions revealed that the 45-nt-long flaA 5’UTR can fold into two stem-loops (SL1 and SL2), both of which harbour an ANGGA motif in their loops (Fig. 2a). The second ANGGA motif 

Figure 2 | CsrA represses flaA translation by binding to its 5’UTR. (a) Predicted secondary structure of the flaA leader using Mfold74. Blue bars indicate GGA motifs; grey: SD sequence. Black triangles indicate RNase T1 cleavages from the structure probing in c. (b) Western blot quantification (n = 5 biological replicates) of FlaA with a C-terminal 3xFLAG epitope tag integrated at its native locus (FlaA-3xFLAG) and northern blot analysis of flaA mRNA (n = 3 biological replicates) in ΔcsrA and various flaA 5’UTR mutant strains. Shown is the mean ± s.e.m (**P < 0.01 using Student’s t-test, NS: not significant). Mutations are depicted in red in a. (c) Gel-shift assays using ~ 0.04 pmol in vitro-transcribed and 5’ end-labelled flaA leader (~ 45 to +99 relative to the start codon) with increasing concentrations of CsrA. (d) Affinity binding curves determined by gel-shift assays for 32P-labelled flaA WT and mutant leaders (~4 nM) based on three replicates. The inset represents an enlargement of the binding curves for low CsrA concentrations. Shown is the mean ± s.d. (e) Footprinting assays of ~0.2 pmol 32P-labelled flaA WT and flaA M2/M3 mutant leaders in the absence or presence of increasing CsrA concentrations (molar excess of 0, 20, 50 and 100 CsrA) using RNase T1. Untreated flaA leader alone or incubated with 100-fold excess of CsrA served as controls and RNase T1- or alkali (OH)-digested flaA leader as ladders, respectively. Blue lines: GGA motifs; green lines: protection from RNA cleavage upon addition of CsrA. The secondary structure of the flaA leader according to a is depicted on the right.
covers the ribosome-binding site and a third GGA is present as the second codon. The flaA 3'UTR secondary structure is conserved and supported by compensatory base-pair changes in other Campylobacter species (Supplementary Figs 6 and 7, and Supplementary Methods). A chromosomally 3xFLAG-tagged FlaA was ~3-fold upregulated in a ΔcsrA strain compared with WT on western blots (Fig. 2b and Supplementary Fig. 8a, lanes 1 and 2). To show that CsrA affected translation by binding to the flaA leader, we introduced chromosomal point-mutations into the two putative GGA CsrA-binding motifs (M1: SL1-GGA->AAA, M2: SL1-GGA->UGG, and M3: SL2-GGA->GGG) Fig. 2a,b and Supplementary Fig. 8a, lanes 3–8). Like deletion of csrA, mutation of the GGA motifs resulted in two- to threefold elevated FlaA-3xFLAG protein expression. FlaA-3xFLAG levels were not affected by deletion of csrA in the flaA leader mutants, indicating CsrA binding was abolished in these strains. Northern blot analysis showed flaA-3xFLAG mRNA levels are only mildly affected in the different mutant strains, further indicating post-transcriptional regulation of flaA by CsrA (Fig. 2b and Supplementary Fig. 8a, lanes 3–8).

In vitro gel-shift assays using recombinant C. jejuni CsrA-Strep and T7-transcribed, 5'-end radiolabelled flaA WT leader showed strong CsrA binding (Kd = ~50 nM) with two defined shifts, indicating at least two CsrA-binding sites (Fig. 2c). In contrast, flaA leaders with GGA point-mutations in either SL1 (M1 and M2), SL2 (M3) or both SL1 and SL2 (M2/M3) showed four- to tenfold higher Kd values (200–500 nM), confirming that the mutations reduced CsrA binding (Fig. 2d and Supplementary Fig. 9a). To map CsrA-binding sites on the flaA leader, we performed in-vitro footprinting assays with labelled flaA leader in the absence or presence of CsrA using enzymatic and chemical cleavage (RNase T1; single stranded G-residues and lead(II) acetate; single-stranded RNA). Cleavage patterns without CsrA confirmed the predicted flaA leader structure (Fig. 2e and Supplementary Fig. 8b). A clear protection was observed at the SL1 and SL2 GGA motifs of the WT leader upon addition of increasing CsrA amounts, but not for a flaA M2/M3 mutant with disrupted binding motifs. The third GGA downstream of the start codon was not protected. Overall, our data suggest C. jejuni CsrA represses flaA translation by high-affinity binding to the two GGA-containing stem-loops SL1 and SL2 in the flaA leader.

The flagellar assembly factor FliW binds CsrA in C. jejuni. The constitutive expression of CsrA during routine culture (Supplementary Fig. 1) suggested modulation of its activity rather than its expression. Because homologues of the CsrB/C sRNAs are absent in C. jejuni, we hypothesized that other RNAs, or even proteins, might control CsrA activity in Campylobacter. One candidate (GJ1075, 129 aa) is a potential homologue of the flagellar assembly factor, FliW, which has a role in motility but is otherwise uncharacterized. In B. subtilis, FliW binds CsrA and antagonizes CsrA-mediated translational repression of hag mRNA, encoding the major flagellin. FliW can also bind Hag, which accumulates in the cytoplasm before flagellar hook completion. Hag thus sequesters FliW from CsrA, allowing CsrA to repress Hag synthesis. Upon completion of the hook, Hag is secreted, FliW is released and CsrA repression of flaA translation is relieved. Thus, this Hag-FliW-CsrA partner-switch mechanism ensures appropriate temporal flagellin synthesis. In Epsilonproteobacteria, ΔfliW homologues are present, but, unlike Bacillus, are not encoded adjacent to csrA (Fig. 3a). To investigate whether FliW can interact with CsrA and FlaA in C. jejuni, we performed protein–protein coIP experiments using chromosomal C-terminal 3xFLAG-tag fusions as bait. The anticipated interaction partners were tagged with mCherry at their C-terminus to allow detection by western blotting. In a FliW-3xFLAG-coIP, CsrA-mCherry was successfully co-purified, indicating the two proteins can interact (Supplementary Fig. 10). Similarly, FliW-mCherry was co-purified in a FlaA-3xFLAG-coIP, indicating conserved interactions between all three proteins. As control, none of the proteins were co-purified in coIPs with strains that carry the mCherry-fusion proteins but not the FLAG-tagged proteins.

FliW antagonizes CsrA-mediated translational repression. To determine whether the FliW–CsrA interaction could antagonize CsrA function in Epsilonproteobacteria, we used FlaA protein levels as a read-out for CsrA activity (Fig. 3b). Whereas FlaA-3xFLAG was ~3-fold upregulated in ΔcsrA, deletion of fliW led to ~6-fold downregulation, consistent with further repression of flaA translation by additional CsrA released upon deletion of its protein antagonist (Fig. 3b). A ΔcsrA/ΔfliW double deletion confirmed that the observed downregulation was indeed mediated through CsrA, as FlaA-3xFLAG levels increased back to those in the ΔcsrA mutant. Despite strong reduction of FlaA-3xFLAG protein levels, a ~2-fold higher flaA mRNA level was observed upon deletion of fliW, indicating additional effects of FliW on flaA expression (Supplementary Fig. 11a). Thus, we constructed a transcriptional reporter composed of the unrelated GJ1321 5'UTR and its early coding region (GJ1321_mini) under the control of the flaA promoter. This reporter was, like the endogenous flaA mRNA, ~2-fold upregulated in the ΔfliW mutant (Supplementary Fig. 11b). As GJ1321 is independent of CsrA-mediated control, FliW seems to have a negative effect (direct or indirect) on flaA transcription.

To uncouple transcriptional control of flaA from its translational regulation, we replaced the σ28-dependent flaA promoter in the FlaA-3xFLAG strain with a constitutive σ70-dependent metK promoter. Upon deletion of csrA in this strain, a ~3-fold increase in FlaA-3xFLAG level was observed, further confirming post-transcriptional regulation of FlaA-3xFLAG protein expression by CsrA (Fig. 3b). Like for the strain expressing FlaA-3xFLAG from its native promoter, FlaA-3xFLAG expressed from the metK promoter was strongly downregulated upon deletion of fliW and was restored to ΔcsrA levels in the ΔcsrA/ΔfliW double mutant. This further indicates FliW antagonizes CsrA-mediated translational repression of flaA in a promoter-independent manner. In addition, decreased flaA mRNA stability was observed upon fliW deletion in rifampicin stability assays. This is consistent with increased translational repression of flaA in the absence of fliW, despite overall higher steady-state flaA mRNA levels because of FliW-dependent increased transcription (Supplementary Fig. 11c).

In line with strong downregulation of the FlaA protein upon fliW deletion, transmission electron microscopy revealed shorter flagella on ΔfliW bacteria compared with those of the WT strain (Fig. 3c,d). In fact, the flagella of ΔfliW appeared similar to those of a ΔfliA mutant strain and of bacteria lacking σ28 (ΔfliA), required for flaA transcription. In contrast, the ΔcsrA and ΔcsrA/ΔfliW strains expressed normal flagellar filaments. The short flagella of the ΔfliW strain are probably composed mainly of the minor flagellin Flab, which is transcribed from an RpoN (σ28)-dependent promoter. Upon deletion of both flagellin genes (ΔflaA/ΔflaB), the bacteria no longer had filaments but the hook structure was visible at the poles (black arrowheads, Fig. 3c). Furthermore, a ΔrpoN mutant strain had neither flagella nor hooks. Motility assays revealed that the ΔcsrA or ΔfliW strains showed a halo-radius reduction to 78% and 72% of WT, respectively (Fig. 3d). Likely due to its shorter flagella, ΔfliW also showed slower autoagglutination than WT, but greater than
the non-motile ΔfliA and ΔrhoN mutants (Supplementary Fig. 12). Overall, these data suggest that, besides a mild effect on flaA transcription, FliW affects post-transcriptional control of FlaA, and therefore filament assembly and motility, in a CsrA-dependent manner.

Expression of flagellar mRNAs is not affected in ΔcsrA. Besides flaA mRNA, many other flagellar targets, such as the 5’UTRs of flaG, flaB and flgI, were strongly enriched in the CsrA-3xFLAG-coIP (> 346-, > 58- and > 170-fold, respectively; Table 1). The flaG, flaB and flgI leaders also have one or more GGA-containing motifs near their SD (Fig. 4a). In vitro gel-shift assays of in vitro transcribed flaG, flaB and flgI leaders, and several other co-purified flagellar mRNAs (Gj0040, flaA and flgM), confirmed CsrA binding (Fig. 4b and Supplementary Fig. 9b). The non-enriched Gj1324 mRNA, encoding a gene involved in flagellin modification, or an unrelated mRNA fragment from H. pylori did not shift with CsrA, confirming specific binding of CsrA to coIP-enriched transcripts (Supplementary Fig. 9c). However, CsrA affinity for flaG, flaB and flgI leaders was lower (Kd = 350 nM) than for the flaA WT leader (Kd = ~ 50 nM, Fig. 4b). Although FlaA-3xFLAG was upregulated upon csrA deletion (Fig. 2b), chromosomally tagged FlaG-3xFLAG, Flab-3xFLAG and Flgl-3xFLAG levels did not change substantially (Fig. 4a).

FliW and flaA mRNA titrate CsrA-mediated repression. The observed strong CsrA-mediated regulation of flaG, flaB and flgI in the E. coli reporter system (Supplementary Figs 4 and 5) indicates that CsrA can, in principle, regulate these targets. Thus, we hypothesized that FliW, or even abundant mRNAs, might sequester CsrA under the examined routine growth conditions, obscuring any regulatory effect on these low-affinity targets. Because flaA mRNA is highly abundant14 and expressed at the end of the flagellar cascade, we reasoned flaA mRNA might itself...
Figure 4 | CsrA binds to other flagellar target mRNAs but csrA deletion does not affect their translation. (a) (Left) Predicted secondary structures of flaG, flaB and flgI leaders using MolI22 with putative GGA binding-sites of CsrA (blue) and SD sequences (grey). (Right) Western blot analyses of FlaG-3xFLAG, FlaB-3xFLAG and FlgI-3xFLAG in C. jejuni WT or ΔcsrA strains. (b) CsrA-binding affinities of flagella mRNA leaders (≤4 nM) determined by in vitro gel-shift assays. The inset represents an enlargement of the binding curves for low CsrA concentrations. Shown is the mean ± s.d.

Figure 5 | The flaA 5'UTR and fliW inhibit CsrA-mediated regulation of flagella genes. Quantification of FlaG-3xFLAG, FlaB-3xFLAG and FlgI-3xFLAG levels using western blot of the indicated C. jejuni NCTC1168 strains grown to mid-log phase (M1: GGA → AAA in SL1 of flaA 5'UTR). Values were calculated based on at least three biological replicates. Shown is the mean ± s.e.m (*P<0.05, **P<0.01, ***P<0.001, using Student’s t-test). NS, not significant.

titrate CsrA activity. To investigate the role of FliW and the flaA mRNA as CsrA antagonists, we analysed FlaG-3xFLAG, FlaB-3xFLAG and FlgI-3xFLAG protein expression in loss-of-function strains of both antagonists. In line with FliW acting as a general CsrA antagonist that limits CsrA activity, deletion of fliW led to a ~3-fold decrease in FlaG-3xFLAG level, which was restored to WT level in a ΔcsrA/ΔfliW double mutant (Fig. 5 and Supplementary Fig. 13a).

Because flaG and flaA are primarily transcribed from σ28-dependent promoters28 and are thus expressed at the same time, monitoring FlaG-3xFLAG might reveal the potential role of flaA 5'UTR as a CsrA antagonist. The chromosomal M1 flaA leader mutation (GGA → AAA in SL1, Fig. 2a), which leaves the coding region intact but abolishes CsrA binding (Fig. 2d), decreased FlaG-3xFLAG levels ~3-fold (Fig. 5 and Supplementary Fig. 13a). Upon introduction of ΔcsrA, FlaG-
3xFLAG expression was restored to WT levels, indicating decreased FlaG expression in the flaA-M1 mutant is dependent on CsrA, and suggesting that the flaA leader can also titrate CsrA. Combining both ΔfliW and flaA-M1 led to a tenfold reduction in FlaG-3xFLAG levels, showing their cumulative effect in antagonizing CsrA. In line with this, the M1/ΔfliW/ΔcsrA triple mutant restored FlaG-3xFLAG levels back to WT levels (Fig. 5 and Supplementary Fig. 13a). Growth curves showed that there was no major impact on growth of the individual mutations under the examined conditions (Supplementary Fig. 13b). Although the ΔfliW and M1/ΔfliW mutants showed a slightly increased growth rate compared with WT, this increase was less than a non-motile ΔfliA strain.

To further confirm the role of the flaA 5’UTR as a CsrA antagonist, a ~250-nt long flaA_mini transcript comprising the flaA leader and first 17 codons followed by a stable ribosomal rrrB terminator was ectopically expressed from the native flaA promoter (Supplementary Fig. 14a). Expression of the flaA_mini transcript in a ΔfliW mutant, which has strong CsrA-mediated flaA translational repression, increased FlaA-3xFLAG levels around 2.6-fold (Supplementary Fig. 14b). This indicates flaA_mini can bind and antagonize CsrA and partially relieve CsrA-mediated repression of flaA translation. A smaller, yet significant, complementation of the effect of a fliW deletion was also observed for FlaG-3xFLAG levels.

Next, the effect of the two antagonists on CsrA-mediated regulation of the RpoN-dependent genes flaB and flgl was evaluated. A similar, yet less pronounced effect compared with FlaG-3xFLAG, was observed for FlaB-3xFLAG upon single or double mutations of fliW and M1. In contrast, FlgI-3xFLAG levels were only significantly reduced upon fliW deletion (Fig. 5 and Supplementary Fig. 13a). Overall, this reveals FliW as the major CsrA antagonist under the examined growth conditions that titrates, along with the flaA mRNA antagonist, CsrA from lower affinity flagellar targets such as flaG.

**flaA mRNA localizes to the poles of elongating cells.** As flaA mRNA can titrate CsrA activity, we wondered when flaA mRNA levels change to modulate CsrA activity. Expression of flaA mRNA appeared constitutive during growth (Supplementary Fig. 13c). However, in the amphitrichously flagellated *C. jejuni*, after every cell division, a new flagellum has to be synthesized at the new pole of each daughter cell. As bacteria in batch culture are not synchronized in cell cycle, differences in flaA mRNA expression might be obscured because of the population-based northern analysis. To monitor flaA mRNA expression in single bacteria, we performed RNA-FISH (fluorescence in situ hybridization) in fixed *C. jejuni* cells from exponential phase. Although the control RNA, 16S rRNA (Fig. 6a, green), was visible in all

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**Figure 6 | flaA mRNA localizes to the poles of shorter cells.** (a) RNA-FISH analysis of 16S rRNA (FITC-labelled DNA oligonucleotide probe, green) and flaA mRNA (14 Cy5-labelled single-stranded DNA oligonucleotide probes, red) in WT cells in mid-log phase using confocal microscopy (scale bar, 1 μm). (b) A magnified RNA-FISH image showing the distribution of fluorescence signals. flaA mRNA (Cy5) and 16S rRNA (FITC) signals were quantified along the long axis length of bacteria using ImageJ software and were subsequently merged as shown at the bottom of the panel (scale bar, 1 μm). The length of individual cells was also quantified using ImageJ. Statistical analysis for average flaA mRNA and 16S rRNA signals over the cell length is provided in Supplementary Fig. 15. (c) Average *C. jejuni* WT cell lengths in bacteria where flaA mRNA is localized (56 cells) or non-localized (85 cells), ***P<10^-15 using Student’s t-test.
cells, flaA mRNA (Fig. 6a, red) was detected in only some of the cells. As a negative control, we also performed flaA mRNA FISH on a ΔfliW mutant strain (Fig. 7a), which showed no expression of flaA at the cell poles in ~20% of WT cells (Fig. 6a,b). Quantification of cell length across the population showed that cells with localized flaA mRNA were significantly shorter than cells without flaA expression (Fig. 6b,c). Live-cell imaging of a non-motile C. jejuni strain (ΔfliA) over two or three division cycles showed regular patterns of an increase in cell length until cells divide at mid-cell, resulting in short daughter cells (Supplementary Fig. 16). This indicates shorter cells likely correspond to cells that have divided and are elongating. Together, these data suggest differential expression of flaA mRNA during the cell cycle and accumulation in elongating cells at the required site of its encoded protein.

**FliW impacts flaA mRNA localization via CsrA.** To investigate whether CsrA-FliW impacts flaA mRNA localization, we next performed RNA-FISH in ΔfliW, ΔcsrA and ΔfliW/ΔcsrA mutant strains. Although csrA deletion had no effect on flaA localization, it was completely abolished in a ΔfliW mutant (Fig. 7a). Instead of a polar localization, flaA mRNA was now dispersed throughout the cell. The loss of flaA mRNA localization upon fliW deletion was not due to lower transcript abundance as its mRNA level is increased despite strong repression at the protein level (Supplementary Fig. 11a). Strikingly, flaA mRNA localization was restored to the cell poles in the ΔfliW/ΔcsrA double mutant, showing CsrA affects localization of flaA mRNA. As a further confirmation of flaA mRNA localization, we performed super-resolution imaging of flaA mRNA FISH in WT and mutant strains using direct stochastic optical reconstruction microscopy (dSTORM)39, which has only recently been applied for bacterial RNA localization30. dSTORM analysis fully supported and complemented the observations from confocal microscopy analysis (Fig. 7b and Supplementary Fig. 17). Overall, this suggests a model where flaA translation is required for polar localization: upon deletion of fliW, CsrA is released and in turn strongly represses flaA mRNA translation to impede its localization to the poles.

**Polar flaA mRNA localization requires its translation.** To support the translation-dependent model of flaA localization, we constructed several point mutants in the native flaA gene that either maintain or disrupt flaA translation (Fig. 8a). Mutation of the start codon of flaA (AUG → AAG (X1) or AUU (X2)) to abolish translation initiation resulted in dispersed flaA mRNA (Fig. 8b). In contrast, when the start codon was changed to an alternative start codon (AUG → GUG (X3)), flaA mRNA still localized to the cell poles, indicating translation of flaA mRNA is indeed required for polar localization. Mutation of the third flaA codon to a stop codon (UUU → UAG (X4)) also resulted in a completely dispersed flaA mRNA signal (Fig. 8b and Supplementary Fig. 17). In contrast, flaA mRNA with a synonymous silent mutation (UUU → UUC (X5); both encoding Phe)

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**Figure 7 | CsrA and FliW influence flaA mRNA localization to the poles.** (a) RNA-FISH analysis (Left: confocal microscopy images; Right: averaged fluorescence intensity along the long axis based on 10 cells) of 16S rRNA (green) and flaA mRNA (red) in C. jejuni NCTC11168 WT, ΔcsrA, ΔfliW, ΔcsrA/ΔfliW and ΔfliA strains in mid-log phase. FITC and Cy5 channels were merged in the microscopy images in the third lanes (scale bar, 1 μm). (b) Super-resolution microscopy imaging of flaA mRNA RNA-FISH (14 Cy5-labelled oligos) in the indicated C. jejuni strains using dSTORM imaging. Cell boundaries from bright-field images are depicted by white dotted lines.

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at the third codon localized similarly to the WT mRNA. Some of
the mutations that abolish translation (X1, X2) lead to reduced
(50–80% of WT) mRNA levels (Supplementary Fig. 18).
Nonetheless, as the strain expressing the mRNA with a stop
mutation at the third codon (X4), which also showed abolished
polar mRNA localization, had even higher (~170%) mRNA
expression levels than WT, it is unlikely that reduced (or
increased) mRNA levels lead to loss of localization. To
determine the effect of terminating translation at a downstream
position, we introduced a stop codon at the 101st codon of
mRNA (CAA > UAA (X6)). This mutant showed partial polar mRNA localization, suggesting the N-terminal peptide might be

**Figure 8** | Translation is required for **flaA** mRNA localization to the cell poles. (a) Point mutations in **flaA** mRNA that were introduced at the native **flaA** locus. Mutations X1, X2, X4 and X6 abolish or prematurely stop **flaA** translation, whereas X3 and X5 represent silent mutations. (b) RNA-FISH analysis (Left: confocal microscopy images; Right: averaged fluorescence intensity along the long axis based on 10 cells) of C. jejuni point mutant strains depicted in a. FITC and Cy5 channels were merged in the third rows of the microscopy images (scale bar, 1μm).
required for recruiting flaA mRNA to the cell poles. Overall, these data support a role of the FliW/CsrA post-transcriptional network in controlling translation-dependent polar flaA mRNA localization in C. jejuni.

Discussion

Using genome-wide RIP-seq, we have identified direct RNA targets of the translational regulator CsrA in a bacterium that lacks the canonical antagonizing sRNAs. Our study revealed the major flagellin mRNA is both the main CsrA target and a dual-function mRNA, which can titrate CsrA activity together with the FliW protein, the main CsrA antagonist (Fig. 9). Compared with microarray-based transcriptome analyses of csrA loss-of-function strains31,32, which might reveal indirect effects or miss targets because of a lack of changes in target mRNA levels despite translational repression, a coIP approach facilitates the identification of direct targets and binding sites. Sanger sequencing of cDNAs from an RsmA-coIP identified six target mRNAs in P. aeruginose32. RNA-seq of a CsrA-coIP in E. coli revealed 721 co-purified transcripts33, and in vivo ultraviolet crosslinking combined with RNA-seq (CLIP-seq) revealed 467 potential CsrA-binding sites in Salmonella typhimurium, including binding sites in many virulence mRNAs34. In our RIP-seq approach, we used untagged WT strains as a negative control to allow for elimination of non-specifically bound transcripts. Our peak-detection tool confirmed the high specificity of this approach, as it detected an ‘ANGGA’ consensus-motif determined by in-vitro selection25. Besides canonical binding to 5’UTRs or early codons5,35, our coIP also revealed CsrA binding within coding regions or between genes in polycistrons to mediate discordant operon regulation.

Our coIP approach revealed many mRNAs of flagellar genes as direct CsrA targets. The motility defect of ΔcsrA suggests that tight regulation of flagellar genes by CsrA, and especially of the major flagellin FlaA, is required for proper motility. Balancing CsrA activity through the antagonizing protein FliW also appears crucial for flagellar assembly, as we observed that a C. jejuni NCTC11168 ΔfliW mutant expresses short flagella, as also reported in other strains27,28, and is defective for autoagglutination and motility in both B. subtilis and C. jejuni39,40. Although CsrA impacts motility by directly controlling flagellin expression in C. jejuni, B. subtilis and Borrelia, the strong motility defect of an E. coli csrA mutant8,41 is due to a requirement of CsrA for stabilization of the mRNA encoding the master regulator FlhDC38. The flagellum also plays an essential, multi-factorial role in C. jejuni colonization and pathogenesis, including secretion of Cia/Fed effectors29,39, and is required for proper cell division40. Future studies might reveal CsrA-affected phenotypes beyond motility.

Instead of CsrA-activity control by antagonizing sRNAs5, we demonstrated that the flaA mRNA itself can titrate CsrA. This represents a new mode of CsrA activity control by a target mRNA-derived antagonist. The flaA mRNA probably only binds one CsrA dimer42. Whereas flaA mRNA probably only binds one CsrA dimer, multiple RsmE dimers are cooperatively assembled on RsmZ sRNA8,41. CsrA titration by a 5’UTR has recently been shown to mediate hierarchical control of fimbriae expression in Salmonella typhimurium13. The fimAICDHF mRNA leader, which in contrast to flaA mRNA is not itself a CsrA target, cooperates with the CsrB/C sRNAs to antagonize CsrA-mediated activation of plasmid-encoded fimbriae. Small RNAs other than CsrB/C can also sequester CsrA in addition to functioning as antisense RNAs44. Global approaches such as RIP-seq are ideally suited to identify additional antagonizing sRNAs or members of the emerging class of dual-function, cross-regulating mRNAs2,3.

Analysis of flaA mRNA expression in single bacteria using RNA-FISH showed that this transcript localizes to the poles of shorter, and presumably elongating, cells. As a new flagellum is synthesized after each cell division at the new pole of the amphitrichous C. jejuni, polar flaA mRNA localization might facilitate this process. This temporal and spatial modulation of flaA mRNA expression might also affect CsrA-mediated regulation of other flagellar genes through mediating varying levels of this CsrA RNA antagonist. Mutations that either abolish or maintain translation showed flaA translation is required for its polar localization. Bacterial mRNA localization has only recently been described and unlike eukaryotes the underlying mechanisms and regulation of this process are poorly understood15,46. Besides co-translational targeting of mRNAs to the required sites of their encoded products, translation-independent mechanisms of RNA localization have also been described20,21, including spatial expression according to chromosome organization. We observed that a flaA mRNA variant with a premature stop-codon mutation at the 101st codon partially localizes, suggesting a role of the N-terminus in directing the nascent peptide along with the mRNA to the secretion apparatus. Little is known how flagellar substrates are selected for secretion, as they do not share a secretion-signal sequence or cleavable signal peptide. N-terminal domains are required for secretion of flagellar proteins in diverse bacteria, including C. jejuni36, and both 5’UTR and N-terminal peptide secretion signals have been shown to contribute to secretion efficiency47. In addition, flagellar
chaperones play a role in regulating the coupling of translation to secretion of flagellar substrates. In Yersinia, cis-encoded RNA localization elements in the early coding region are required for secretion of effector proteins by type III secretion systems. Future studies will identify and clarify the role of elements, either in the protein N-terminus or the mRNA 5′UTR, as well as potential interaction partners that are crucial for directing the peptide and/or mRNA to the cell poles and secretion apparatus. Besides the requirement of flaA translation for localization, other factors such as the flaA genomic location or the translational complex might also contribute to polar flaA mRNA localization.

Our study revealed an unexpected function for the CsrA-FliW network in spatial and temporal gene-expression control, and specifically FliW affects translation-dependent polar localization of the flagellin mRNA by antagonizing CsrA-mediated translational repression. The limited CsrA activity in WT cells under standard growth conditions, because of sequestration by the FliW protein antagonist, probably allows sufficient translation of flaA mRNA for its polar localization. Strong CsrA-mediated translational repression of flaA upon fliW deletion is probably responsible for the diffuse flaA localization, while the FliW mutant or CsrA binding might mediate storage of translationally inactive mRNA until synthesis of FlaA is required or proper localization is achieved, similar to mRNP granules in eukaryotes. Future studies will show whether other flagellar mRNAs also polarly localize and if the CsrA-FliW regulatory network also impacts their localization. CsrA-mediated regulation of mRNA localization might also occur in B. subtilis and B. burgdorferi, where CsrA overexpression represses the major flagellin. An analogous system might have also evolved in the Alphaproteobacterium Caulobacter crescentus, which encodes two proteins with opposing activities on flagellin regulation, FlaF and FlbT, whereby FlbT post-transcriptionally regulates flagellin mRNA for its polar localization. Strong CsrA-mediated translational repression. The limited CsrA activity in WT cells under standard growth conditions, because of sequestration by the CsrA antagonist affects other flagellar genes.

**Methods**

**Bacterial strains, oligonucleotides and plasmids.** All C. jejuni and E. coli strains used in this study are listed in Supplementary Table 3 and DNA oligonucleotides in Supplementary Table 4, respectively. Plasmids are summarized in Supplementary Table 5.

**Bacterial growth conditions.** C. jejuni strains were routinely grown on Müller-Hinton agar plates or with shaking in Brucella broth (BB), both supplemented with 10 μg ml⁻¹ vancomycin, at 37°C under microaerobic (10% CO₂, 5% O₂) conditions as described previously. The agar was further supplemented with marker-selective antibiotics (20 μg ml⁻¹ chloramphenicol, 50 μg ml⁻¹ kanamycin, 20 μg ml⁻¹ gentamicin or 250 μg ml⁻¹ hygromycin B) where appropriate. E. coli strains were grown aerobically at 37°C in Luria-Bertani (LB) medium supplemented with appropriate antibiotics. For induction of arabinose-inducible pBAD promoter, 0.001% (+) or 0.0005% (+ +) L-arabinose was added to LB media.

**Construction of bacterial mutant strains.** All C. jejuni mutant strains (deletion, chromosomal 3xFLAG-tagging, chromosomal point mutations) were constructed using double-crossover homologous recombination. Cloning strategies and the generation of constructs are described in detail in the Methods and Supplementary Methods. Oligonucleotides used to amplify regions of upstream/downstream sites, respectively. Following cleanup, the PCR product was digested with EcoRI and XbaI and ligated into a similarly digested pGG1 backbone, generated by inverse PCR with primers CSO-0074/-0075, to create plasmid pGD2-1. The plasmid was confirmed by colony PCR with primers JVO-0054/CSO-0173 and the sequence was verified by colony PCR with primers JVO-5142 and HPK2. The ‘C-term’ region of flaG, flaA, flagG and flaB proteins were fused to a 3xFLAG epitope at their C-termi

**Construction of 3xFLAG epitope-tagged proteins in C. jejuni.** C. jejuni genes were chromosomally tagged at their C-termi

**3xFLAG tagging of proteins by overlap PCR.** Construction of a C-terminal 3xFLAG translational fusion at its native locus was performed by overlap PCR for flaG as described in Supplementary Methods for gene deletions, but with the following modifications. The final overlap PCR product contained ~500 bp of the C-terminal coding region of flaG minus the stop codon (C-term) and ~500 bp downstream of flaG (DN) for homologous recombination. These regions flanked an in-frame 3xFLAG tag and stop codon followed by an aphA-3 Kan cassette. For example, for tagging flaG, the 3xFLAG tag and Kan cassette was amplified from plasmid pGG1 with primers JVO-5142 and HPK2. The ‘C-term’ region of flaG was amplified using primers CSO-1002/-1003, where CSO-1002 is sense to DN and contains a region of CsrA titration by FliW indicates that CsrA-activity control by a protein antagonist, a mechanism first identified in the Gram-positive B. subtilis, is more widespread than previously appreciated. Besides the post-transcriptional effect of FliW on flaA and other flagellar genes by antagonizing CsrA, deletion of fliW directly or indirectly increases flaA transcription. Transcription of hag is also twofold upregulated in B. subtilis upon fliW deletion. Although FliW appears to be the main CsrA antagonist, its synergistic interplay with the flaA mRNA antagonist affects other flagellar genes showed that RNA-based regulation can also impact CsrA activity in this type of Csr network. Gammaproteobacterial genomes encode CsrA as well as the antagonizing sRNAs and anti-correlation between the presence of the CsrB/C sRNAs and FliW has been observed. As the csrA gene is located next to a tRNA cluster in E. coli, this strongly suggests the pleiotropic function of CsrA in Gammaproteobacteria might have been horizontally acquired, followed by evolution of the antagonizing sRNAs. Thus, the conserved or possibly more ancient function of the CsrA-FliW system might be to mediate temporal and spatial control of proper flagellum assembly. During our conservation analysis we observed that certain non-flagellated Campylobacter species, such as C. hominis, C. gracilis and C. ureolyticus, lack csrA and fliW homologues, further supporting their conserved function in flagellar regulation. Further studies are required to unravel the full complexity of the CsrA-FliW regulatory network and its impact on RNA localization.
were checked by colony PCR with primers CSO-1005/HPK2 and western blot analysis with an anti-FLAG antibody.

Introducing chromosomal point mutations into the flaA leader. To introduce point mutation into the 5′UTR of flaA at the native locus, a 1.10-bp deletion from the flaA promoter was amplified using oligos CSO-0752/-0753. These primers introduced Xhol and XhoI sites, respectively, into the resulting PCR product. After Xhol and XhoI digestion, the product was then ligated into a similarly digested plasmid pTV7-1, resulting in plasmid pGD70-5. Plasmid pGD70-5 was checked by colony PCR using primers pZE-A/CSO-0753 and sequencing with pZE-A. Next, plasmid pGD70-5 was amplified by inverse PCR using primers CSO-0754/-0755, thereby introducing Ndel and BamHI restriction sites 40 nt upstream of the flaA transcriptional start site (TSS). An in(3)-IV gentamicin resistance cassette with its own promoter and terminator was inserted using Gt3I and Gt3II amplified pGD70-5 in the reverse orientation to flaA, just upstream of its promoter, using the Ndel/BamHI restriction sites, resulting in plasmid pGD76-1. Plasmid pGD76-1 was checked by colony PCR using primers CSO-0576/-0753 and sequencing with CSO-0753.

Point mutations were then introduced into the flaA 5′UTR by inverse PCR on pGD76-1 using complementary oligos harbouring the desired mutation, followed by DpnI digestion and transformation of the resulting purified PCR product into E. coli TOP10. For introduction of the flaA M1 mutation (GGA > AAG in stem-loop SL1 of the flaA leader), oligonucleotides CSO-1114/-1115 were used for PCR on pGD76-1. The mutation was confirmed in the resulting plasmid pGD92-1 by sequencing. Similarly, the flaA M2 (GGA > GGA in stem-loop SL1 of the flaA leader), M3 (GGA > GGG in stem-loop SL2 of the flaA leader), X1 start codon (AUG > AAG), X2 start codon (AUG > AUA), X3 start codon (AUG > AUG), X4 3′ end codon (UUU > UAG), X5 3′ end codon (UUA > UUC) and X6 1014 codon (CAA > UAA) mutations were introduced using primers pairs CSO-0757/-0758, CSO-1116/-1117, CSO-2019/-2020, CSO-2827/-2828, CSO-2825/-2826, CSO-2829/-2830, CSO-2831/-2832 and CSO-2833/-2834, respectively, in plasmids pGD77-1, pGD93-1, pGD114-2, pGD205-1, pGD204-1, pGD206-1, pGD207-1 and pGD208-1, respectively. For introduction of the flaA M2 and M3 mutations, a similar mutagenesis approach was performed based on PCR amplification of the M2 plasmid pGD77-1 using oligonucleotides CSO-1116/-1117, resulting in pGD95-1 harbouring both the mutations. To introduce the flaA 5′UTR mutations into C. jejuni, a PCR product covering the homologous ends and the gentamicin resistance cassette was amplified from the respective WT (pGD76-1) or mutant plasmids using CSO-0752/-0850 and electroporated into C. jejuni as described above. To confirm introduction of point mutation in the flaA leader, colony PCR was performed using CSO-0576/-0753 and sequencing with CSO-0850.

Construction of E. coli mutants. The E. coli ΔggAΔ and ΔggAΔΔsrAΔ deletion strains were constructed in the TOP10 background using the Φ red protocol. Briefly, a kanamycin resistance gene, amplified from plasmid pKD4 using primers CSO-0652/-0653, was used to replace the entire ggA ORF excluding the start and stop codon. The mutant strain was verified by colony PCR using the primer pairs CSO-0654/-0655 and CSO-0652/-0655. After verification, helper plasmid pCP20 containing FLP recombinase was introduced to remove the kanamycin resistance marker. The helper plasmid, which is temperature-sensitive and carries an ampicillin resistance marker, was then cured by recovering colonies at 37°C and confirming ampicillin sensitivity, resulting in strain CSO-0552. Similarly, the ORF of the flaA gene was replaced by an ampicillin resistance cassette. For this purpose, the flaA ORF was amplified from E. coli strain SL1 of the flaA leader, containing the leader, oligonucleotides CSO-1114/-1115 were used for PCR amplification of the M2 plasmid pGD77-1 using oligonucleotides CSO-1116/-1117, resulting in pGD95-1 harbouring both the mutations. To introduce the flaA 5′UTR mutations into C. jejuni, a PCR product covering the homologous ends and the gentamicin resistance cassette was amplified from the respective WT (pGD76-1) or mutant plasmids using CSO-0752/-0850 and electroporated into C. jejuni as described above. To confirm introduction of point mutation in the flaA leader, colony PCR was performed using CSO-0576/-0753 and sequencing with CSO-0850.

RIP-seq of C. jejuni CsrA-3xFLAG. colP combined with RNA-seq (RIP-seq) to identify direct RNA-binding partners of CsrA-3xFLAG in C. jejuni was performed as previously described 18,29 with minor modifications.

CoIP of RNA with CsrA-3xFLAG. CoIP of chromosomally epilogue-tagged C. jejuni CsrA with an anti-FLAG antibody and Protein A-Septase beads was performed from lysates of C. jejuni NCTC11168 and 18. Cells were harvested by centrifugation at 15,200g for 2 min. The coIP precipitated the CsrA-3xFLAG tagged library, log2 fold changes (FCs) rather than GO enrichment was used. For determination of genes enriched in the CsrA-3xFLAG-tagged library, coIP reads to annotations was assessed for each library by counting all reads overlapping selected annotations on the sense strand. These annotations consist of strain-specific NCBI gene annotations complemented with annotations of previously determined 5′UTRs and small RNAs 14. Each read with a minimum overlap of 10 nt was counted with a value based on the number of locations where the read was mapped. If the read overlapped more than one annotation, the value was divided by the number of regions and counted separately for each region (for example, one-third for a read mapped to three locations).
Similar analysis was done for strains 81-176 using annotations provided in NC_008787.gff (chromosome), NC_008770.gff (pVir plasmid) and NC_008790.gff (pTel plasmid).

Peak detection and CsrA-binding motif analyses. To automatically define CsrA-bound RNA regions or peaks from the CsrA-3xFLAG coIP data sets, an in-house tool ‘sliding_window_peak_calling_script’ was developed based on a sliding window approach. A detailed description of the tool will be described elsewhere. The script has been deposited at Zenodo (https://zenodo.org/record/49292) under DOI 10.5281/zenodo.49292 (http://dx.doi.org/10.5281/zenodo.49292). The script is written in Python 3 and requires installation of the Python 3 packages numpy and scipy for execution.

In brief, the ‘sliding_window_peak_calling_script’ software uses normalized wiggle files of the CsrA-3xFLAG and control coIP libraries as input to determine sites showing a significant amount of CsrA enrichment compared to the control. The identification of enriched regions is based on four parameters: a minimum required fold change (FC) for the enrichment, a factor multiplied by the 90th percentile of the wiggle graph, which reflects the minimum required expression (MRE) in the tagged library, a window size in nt (WS), for which two values are calculated in a sliding window approach, and a nucleotide step size (SS), which defines the steps in which the window is moved along the genomic axis. All consecutive windows that fulfill the enrichment requirements are assembled into a single peak pile-up. The peak detection is performed separately for the forward and reverse strand of each replicon. For the CsrA-3xFLAG coIP data set, the following parameters were used: FC = 3, MRE = 3, WS = 25 and SS = 5.

For the prediction of consensus motifs based on the peak sequences, MEME64 and CMinder 0.2.1 (ref. 62) were used. For MEME predictions, the following settings were applied: Search 0 or 1 motif of length 4–8 bp per sequence in the given strain and only for the presence of a structural motif. CMinder 0.2.1 (ref. 62) was run on the enriched peak sequences with default parameters except for allowing a minimum single stem loop candidate length of 20 nt. The top-ranked motif incorporated 276 of the 328 sequences and was visualized by R2R63.

Functional classes enrichment analysis. To check for overrepresentation of functional classes of CsrA-bound genes, we considered genes with at least fivefold enrichment in their 5‘UTR and/or coding sequence in the CsrA-3xFLAG library (versus control) as CsrA-bound and the remaining genes as unbound.

We applied an existing functional classification64 of genes from strain NCTC11168 to determine statistically enriched functional classes. Because a similar classification was not available for strain 81-176, a table with orthologue mappings between the two strains was downloaded from OrtholugeDB65 and used to assign the NCTC11168 functional classes to their respective 81-176 counterparts. Genes in our annotation lists without an existing functional classification in NCTC11168 or without an orthologue match were assigned to class 5.1, defined as ‘Unknown’, in our annotation lists without an existing functional classification in NCTC11168 or otherwise. These genes were not included in the functional enrichment analysis.

Protein–protein coIP. The FiW and CsrA protein–protein coIP was performed exactly as described in the RIP-seq coIP protocol (see above) until the step where beads were washed five times with Buffer A. After washing, the beads were suspended in 200 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, 0.1% (w/v) bromophenol blue) and boiled for 8 min. Lysate samples corresponding to an OD600 of 0.05 and 2 (for the second strain) were washed five times with Buffer A. After washing, the beads were collected from SDS–PAGE and immunoblotting with primary antibody at 4°C, membrane was then washed with TBS-T, followed by 1× washing with secondary antibody. After washing, the blot was developed using enhanced chemiluminescence-reagent. GFP-, FLAG-, and Strep-tagged proteins of interest were detected with monoclonal anti-GFP (1:1000 in 3% BSA/TBS-T; Roche, #1181460001), monoclonal anti-FLAG (1:1000 in 3% BSA/TBS-T; Sigma-Aldrich, #F0818-1MG) or monoclonal anti-Strep (1:1000 in 3% BSA/TBS-T; IBA Gmbh, # S-4562-5ML) primary antibody. Mouse IgG (1:10000 in 3% BSA/TBS-T; GE-Healthcare, #RP2401) secondary antibody. mCherry-tagged proteins were detected using a polyclonal anti-mCherry (1:4000 in 3% BSA/TBS-T; Acris, #AB08040-20) primary antibody and an anti-goat anti-rabbit IgG (1:10000 in 3% BSA/TBS-T; Sigma-Aldrich, # GE5632-5ML) and an anti-rabbit IgG (1:10000 in 3% BSA/TBS-T; GE-Healthcare, #RP2401) secondary antibody were used as a loading control. Images of full blots that were cropped in main figures are shown in Supplementary Fig. 19.

Validation of CsrA targets with a GFP reporter system. Validation of CsrA targets was performed using a heterologous E. coli system previously developed for validation of sRNA–mRNA interactions62. Selected candidate C. jejuni CsrA target sequences from the coIP were cloned as translational fusions to GFP or FLAG in plasmids pXG-10 or pXG-30 as listed in Supplementary Tables 3 and 5. Levels of GFP or FLAG translational fusions were then determined by western blotsting or Flowcytometric analysis with E. coli AGP402. AGP402 is a CsrA-deleted strain harbouring plasmid pGD72-3 with C. jejuni CsrA-Strep under the control of an arabinose inducible promoter.

Flow cytometric analysis. For FACS analysis GFP reporter fluorescence in E. coli, cells corresponding to a OD600 were collected from LB cultures in log phase and resuspended in 0.25 ml PBS. Cells were then fixed for 10 min with 0.25 ml of 1% formaldehyde, collected by centrifugation and washed twice with 0.5 ml PBS before final resuspension in 0.5 ml PBS. A 1/100 dilution in PBS was used for measurement. Measurements (50,000 counts per sample) were performed on a BD FACS Calibur machine and analysed using FlowJo (V10).

Purification of C. jejuni CsrA. Recombinant, C-terminal Strep-tagged C. jejuni CsrA (Cl1103) was overexpressed and purified from E. coli TOP10 AgpaAg/AcsrA using Strept–Tactin Sepharose (IBA Gmbh, #2-1202-001). Primers and plasmids used for cloning are listed in Supplementary Tables 4 and 5. The csrA gene, including its 5′UTR, was fused to a C-terminal Strep-tag in the arabinose-inducible plasmid pBAD/Myc-His A (Invitrogen) for overexpression and affinity purification. The csrA-coding region and SD were amplified from C. jejuni NCTC11168 genomic DNA using primers CSO-0746-FW/0747 and the pBAD/Myc-His A plasmid was amplified by inverse PCR with JVO-0900/0901 as previously described63. CSO-0747 and JVO-0901 introduce an XhoI site to the insert and vector, respectively, whereas CSO-0746 has a 5′-phosphate to facilitate blunt-end ligation. XhoI-digested insert and vector were then ligated, resulting in pGD68-1. Plasmid pGD68-1 was checked by colony PCR using primers pBAD-FW/CSO-0747 and sequencing with pBAD-FW. A Strep-tag (WSHPQFEK) was then added to the C-terminus of csrA by inverse PCR using oligonucleotides CSO-0852/0853, resulting in plasmid pGD72-3. Plasmid pGD72-3 was checked by sequencing by pBAD-FW. Plasmid pGD72-3 was then introduced into an E. coli TOP10 AgpaAg/AcsrA deletion strain resulting in strain CSS-0931. CSS-0931 was grown in 500 ml LB broth with 100 μg/ml of ampicillin at 37°C and shaking at 220 r.p.m. to an OD600 of 0.4. The culture was then added to an overnight culture of JVO-0901 containing 100 μg/ml of ampicillin and shaken at 30°C for 16 h. The culture was then diluted in 5 ml of Buffer W (IBA Gmbh, #2-1003-100) and the protocol was followed as per the manufacturer’s instructions using 1 ml Gravity flow–Tactin Sepharose. The majority of CsrA-Strep protein was finally eluted using Buffer E (IBA Gmbh, #2-1000-025) in three successive steps (E1: 0.8 ml, E2: 1.4 ml and E3: 0.8 ml). The majority of CsrA-Strep was concentrated in the E2 fraction. Concentration was quantified using Roti-Quant (Carl ROTH, #K0153), and the protein was stored at −20°C in 50 μl aliquots.

RNA isolation. Bacteria were grown to the indicated growth phase and culture volume corresponding to a total amount of 4 OD600 was harvested and mixed with 0.2 volumes of stop mix (95% ethanol and 5% phenol, vol/vol). The samples were 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. Cell pellets were lysed by resuspension in 600 μl of a solution containing 0.5 mg/ml of lysozyme in TE buffer (pH 8.0) and 80 μl of 10% SDS. The samples were incubated at 1–2 min at 65°C to ensure lysis. After two washes, the RNA was extracted using the hot-phenol method as described previously13,14.

Northern blot analysis. For northern blot analysis, 5–10 μg RNA sample was loaded per lane. After separation on 6% PAA gels containing 7 M urea, RNA was transferred to Hybond–XL membranes (GE-Healthcare) by electroblotting. After blotting, the RNA was ultraviolett cross-linked to the membrane and hybridized with 32P-ATP end-labelled DNA oligonucleotides (Supplementary Table 4).

Rifampicin RNA stability assays. To determine the stability of flta mRNA in C. jejuni NCTC11168 WT, ΔcsrA, ΔflaA and ΔcsrA ΔflaA strains, cells were grown in M9 medium supplemented with 0.05 μg/ml rifampicin and harvested at mid-log phase and treated with rifampicin to a final concentration 500 μg/ml. Samples were harvested for RNA isolation at indicated time points following rifampicin addition (0, 4, 8, 16 and 32 min) as described previously16.
above. After RNA isolation, 10 μg of each RNA sample was used for northern blot analysis as detailed above.

In-vitro T7 transcription and RNA labelling. DNA templates containing the T7 promoter sequence were generated by PCR using oligos and DNA templates listed in Supplementary Table 8. T7 in-vitro transcription of RNAs was carried out using the MEGAscript T7 kit (Ambion) and sequences of the resulting T7 transcripts are listed in Supplementary Table 8. In vitro transcribed RNAs were quality checked and 5' end-labelled (5'P) as previously described46,47.

Gel mobility shift assays. Gel-shift assays were performed using ~0.04 pmol 5’-labelled RNA (4 nM final concentration) with increasing amounts of purified C. jejuni CsrA in 10 μl reactions. In brief, 5’-radio labelled RNA (32P, 0.04 pmol in 6 μl) was denatured (1 min, 95 °C) and cooled for 5 min on ice. Yeast tRNA (1 μg) and 1 μl of 10 x RNA Structure Buffer (Ambion: 10 mM Tris, pH 7, 100 mM KCl, 10 mM MgCl₂) was then added to the labelled RNA. CsrA protein (2 μl diluted 1:1 in 1 x Structure Buffer) was added to the desired final concentrations (0 nM, 10 nM, 20 nM, 50 nM, 100 nM, 200 nM, 500 nM, 1 μM or 2 μM CsrA). Binding reactions were incubated at 37 °C for 15 min. Before loading on a pre-cooled native 6% PAGE, 0.5 x TBE gel, samples were mixed with 3 μl native loading buffer (50% (v/v) glycerol, 0.5 x TBE, 0.2% (w/v) bromophenol blue). Gels were run in 0.5 x TBE buffer at 300 V at 4 °C for 3 h. Gels were dried and analysed using a PhosphorImager (FLA-3000 Series, Fuji).

In vitro structure probing assays. In vitro structure probing of flaA WT and flaA M1/M2 leaders with RNase T1 and lead(II) acetate was performed as previously described48. For each reaction, 0.1 pmol of a labelled flaA leader variant was denatured for 1 min at 95 °C and chilled on ice for 5 min. One microgram yeast tRNA as competitor and 10 x RNA Structure Buffer was added (provided together with RNase T1, Ambion). Unlabelled recombinant CsrA protein (0.1 pmol labelled flaA WT leader RNA in 1 x alkaline hydrolysis buffer (Ambion) for 5 min at 95 °C. Ladders and samples were then separated on 10% (v/v) PAA/7M urea gels in 1 x Structure Buffer for 1 h. Cells were again collected by centrifugation, completely dried in a laminar flow hood and then washed twice with 2 x SSC before final resuspension in 0.5 ml of 2 x SSC containing 10% formamide. Fluorescently labelled DNA oligos (14 Cy5-labelled oligos to detect flaA mRNA and one FITC-labelled oligo specific for 16 S rRNA, Sigma, Supplementary Table 4) were then added at a concentration of 10 ng μl⁻¹ and incubated at 37 °C overnight. The next day, cells were collected by centrifugation and washed three times for 1 h at 37 °C with 0.5 ml of 2 x SSC containing 10% formamide before final resuspension in 2 x SSC (50-250 μl). Cells were then imaged in a Leica Confocal TCS SP5 II microscope using sequential scanning mode.

dSTORM. For super-resolution imaging, C. jejuni cells were grown, fixed and labelled using the above-described RNA-FISH protocol (14 Cy5-labelled DNA oligonucleotides to detect flaA mRNA and a FITC oligo to label 16S rRNA, Sigma, Supplementary Table 4). Labelled cells were immobilized on poly-D-lysine (Sigma-Aldrich)-coated eight-well chambered cover glasses (Sarstedt). For fluorophore photo switching, a buffer with a pH of 8.3-8.5 was used containing 50 mM Tris-HCl (pH 8), 10% glycerol, 1% 2-mercaptoethanol (Carl Roth), 3 μl⁻¹ pyranose oxidase (Sigma-Aldrich) and 90 μM 1-catalase (Sigma-Aldrich) in 2 x SSC.

dSTORM was performed on a wide-field setup for localization microscopy71. An optically pumped semiconductor laser (Genesis MX STM-Series, Coherent) with a wavelength of 639 nm (maximum power of 1 W) was used for excitation of Cy5 and a diode laser (iBeam smart family, TOPTICA Photonics) with a wavelength of 405 nm (maximum power of 120 mW) was used for excitation of Cy3. After the beams were cleaned up for background pass filters (Chroma) and combined by appropriate dichroic mirrors (LasermUX filters, Semrock). Afterwards they were focused onto the back focal plane of the high numerical oil-immersion objective (Olympus APO 60XO TIRF, numerical aperture 1.49), which is part of an inverted fluorescence microscope (Olympus IX71). To separate the excitation light from the fluorescence light, suitable dichroic beam splitters (Semrock) were placed into the light path before the laser beams enter the objective. Fluorescence light collected by the objective was filtered by appropriate detection filters (Semrock/Chroma) and was detected by an EMCCD camera with 512 x 512 pixels (Xon Ultra 897, Andor Technology). The pixel size in the image was 0.19 μm/pixel. Cy5 was excited with the 639-nm laser at a maximum intensity of 4.19 kW cm⁻². During imaging, the 405-nm laser was switched on to keep up a suitable switching ratio. Its laser power was increased successively to a maximum intensity of 0.048 kW cm⁻². For every image, 5,000-25,000 frames were taken with an integration time of 15 ms per frame. For every imaged area, additionally a bright-field image was taken to identify single bacteria. Data analysis was performed using rapiStORM open source software72.

Statistical analysis. All data for western, northern blot or FISH analysis are presented as mean ± s.e.m. Statistical analysis was carried out using Student’s t-test. For statistical comparison of two groups, a two-tailed paired Student’s t-test was used. A value of P < 0.05 was considered significant and marked with an asterisk (*) as explained in the legends. For FISH analysis, fluorescence data curves from 10 cells from a single image were merged as a single averaged curve after cell length normalization. The data were acquired and normalized over cell length using ImageJ and subsequently the merged average curve was generated using Microsoft Excel.

Code availability. The ‘sliding_window_peak_calling_script’ for identification of CsrA-binding sites based on RIP-seq data has been deposited at Zenodo (https://zenodo.org/record/492929). The authors declare that all other data supporting the findings of this study are available within the article and its supplementary information files, or from the corresponding author upon request.

Data availability. The raw, de-multiplexed reads as well as coverage files of the RIP-seq libraries have been deposited in the NCBI Gene Expression Omnibus73. The authors declare that all other data supporting the findings of this study are available within the article and its supplementary information files, or from the corresponding author upon request.
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Author contributions
G.D., S.L.S. and C.M.S. designed the study. G.D., S.L.S., T.B., S.W., M.S. and C.M.S. performed the experiments and analysed the data. R.R. provided reagents and deep sequencing and T.B. performed the RNA-seq data analysis. G.D., S.L.S. and C.M.S. wrote the manuscript with input from all the authors.

Additional information
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