Text S1. Methods for constructing bacterial strains and plasmids used in this study.

Bacterial strains. *C. jejuni* 81-176, a clinical isolate that is able to cause diarrheal disease in human volunteers and promote commensal colonization of avian species, is the wild-type strain that was the source of all genes analyzed in this study (1-3). *C. jejuni* 81-176 *rpsL*^{Sm} Δ*astA* (DRH461), which lacks the gene encoding arylsulfatase, served as the wild-type strain for all experiments in this work (4). Previously described *C. jejuni* 81-176 *rpsL*^{Sm} Δ*astA* mutant strains include: Δ*flgR* (DRH749; (4)); Δ*flgS* (DRH911; (4)); Δ*flhA* (DRH979; (4)); Δ*fliP* (DRH1016; (4)); Δ*flhB* (DRH1734; (5)); Δ*fliE* (SNJ915; (6)); and Δ*flgG* (SNJ925; (7)). Additionally, *C. jejuni* 81-176 *rpsL*^{Sm} Δ*fliR* (DRH755) has been previously described (4). *E. coli* DH5α was used for all cloning procedures. Electroporation of *C. jejuni* and creation of insertional and in-frame deletions were performed as previously described (8, 9).

Construction of *C. jejuni* mutants and transcriptional reporter strains. All strains constructed in this work and plasmids used for construction of mutants are listed in Tables S1 and S2. The *fliN*, *flgB*, *fliY, flgC*, *flgF*, *flgH*, and *flgI* loci with 750 nucleotides of flanking sequence were amplified by PCR from *C. jejuni* 81-176 chromosomal DNA with primers containing 5' BamHI sites (10). These loci were then cloned into BamHI-digested pUC19 to create pDRH275, pDRH1348, pDRH1349, pDRH1878, and pJMB533 or BamHI-digested pBR322 to create pJMB1635. To create a restriction site within *fliY*, pJMB1635 was subjected to PCR-mediated mutagenesis to change nucleotide T132 to an adenine and A133 to a thymine, which generates a SwaI site within *fliY* , resulting in pJMB1971 (11). All plasmids were verified by DNA sequencing. A SmaI-digested *cat-rpsL* cassette from pDRH265 was then ligated into: the EcoRV site of *fliN* in pJMB533 (creating pJMB537); the SwaI site of *fliY* in pJMB1971 (creating pJMB1980); the EcoRV site of *flgF* in pDRH1349 (creating pDRH2534); the EcoRV

site of *flgI* in pDRH1348 (creating pDRH2536), the PmlI site of *flgH* in pDRH1878 (creating pALU101; (8)).

Generation of mutants containing *cat-rpsL* insertions of genes was accomplished by electroporating *C. jejuni* 81-176 *rpsL*^{Sm} Δ*fliR* (DRH1701) with pDRH414 or *C. jejuni* 81-176 *rpsL*^{Sm} Δ*astA* (DRH461) with pDRH1814, pDRH2534, pDRH2536, pJMB537, pJMB572, pJMB1980, pSNJ356, pSMS469, pALU101, and pALU115 (4, 6). Transformants were selected on MH agar containing chloramphenicol and verified by colony PCR. The transformants recovered were: 81-176 *rpsL*^{Sm} Δ*fliR astA*::*cat-rpsL* (DRH1647), 81-176 *rpsL*^{Sm} Δ*astA fliF*::*cat-rpsL* (DRH1876), 81-176 *rpsL*^{Sm} Δ*astA fliQ*::*cat-rpsL* (DRH2305), 81-176 *rpsL*^{Sm} Δ*astA fliG*::*cat-rpsL* (JMB1161), 81-176 *rpsL*^{Sm} Δ*astA fliM*::*cat-rpsL* (JMB1163), 81-176 *rpsL*^{Sm} Δ*astA fliN*::*cat-rpsL* (JMB1407), 81-176 *rpsL*^{Sm} Δ*astA flgI*::*cat-rpsL* (JMB2004), 81-176 *rpsL*^{Sm} Δ*astA fliY*::*cat-rpsL* (JMB2008), 81-176 *rpsL*^{Sm} Δ*astA flgF*::*cat-rpsL* (SNJ931), 81-176 *rpsL*^{Sm} Δ*astA flgB*::*cat-rpsL* (SNJ1043), and 81-176 *rpsL*^{Sm} Δ*astA flgH*::*cat-rpsL* (ALU107).

Plasmids pDRH275, pDRH1349, pDRH1777, pDRH1878, pDRH2407, and pJMB531 were subjected to PCR-mediated mutagenesis to generate in-frame deletions of specific regions of genes (11). For detailed description of in-frame deletions of genes in the respective plasmids, see Table S2. All plasmids were sequenced to verify the correct mutation or deletion. As a result, the following plasmids were created: pDRH2422 (pUC19::Δ*flgH*), pDRH2504 (pUC19::Δ*flgF*), pJMB1204 (pUC19::Δ*fliG*), pJMB1401 (pUC19::Δ*fliM*), pJMB1512 (pUC19::*fliG*$_{\Delta MCD}$), pJMB1513 (pUC19::*fliG*$_{\Delta CTD}$), pJMB1516 (pUC19::*fliF*$_{\Delta G511\text{-}S520}$), pJMB1729 (pUC19::*fliG*$_{\Delta MD}$), pJMB1731 (pUC19::*fliG*$_{\Delta NTD}$), pJMB1827 (pUC19::*fliF*$_{\Delta A176\text{-}S176}$), pSNJ919 (pUC19::Δ*flgB*), and pSNJ1009 (pUC19::Δ*flgC*).

Mutant strains with chromosomal in-frame deletion of genes were generated by electoporating the following combination of strains and plasmids: ALU107 and pDRH2422; DRH1876 and pDRH2073 or pJMB1827; JMB1161 and pJMB1204, pJMB1512, pJMB1513, pJMB1729, pJMB1731, or pJMB2119; JMB1163 and pJMB1401; SNJ1043 and pSNJ919 or pSNJ1009; DRH1647 and pDRH449; DRH2305 and pSMS443; and SNJ931 and pDRH2504 (4, 6). All transformants were recovered on MH agar containing streptomycin and verified by colony PCR. In addition, all transformants were verified for the production of FlgS and FlgR by immunoblotting (12, 13). As a result, the following strains were isolated: DRH1701 (81-176 *rpsL*^{Sm} Δ*astA* Δ*fliR*), DRH2077 (81-176 *rpsL*^{Sm} Δ*astA* Δ*fliF*), DRH2468 (81-176 *rpsL*^{Sm} Δ*astA* Δ*flgH*), JMB1230 (81-176 *rpsL*^{Sm} Δ*astA* Δ*flgF*), JMB1242 (81-176 *rpsL*^{Sm} Δ*astA* Δ*fliG*), JMB1415 (81-176 *rpsL*^{Sm} Δ*astA* Δ*fliM*), JMB1517 (81-176 *rpsL*^{Sm} Δ*astA fliG*$_{\Delta MCD}$), JMB1641 (81-176 *rpsL*^{Sm} Δ*astA fliG*$_{\Delta CTD}$), JMB1748 (81-176 *rpsL*^{Sm} Δ*astA fliG*$_{\Delta MD}$), JMB1756 (81-176 *rpsL*^{Sm} Δ*astA fliG*$_{\Delta NTD}$), JMB1960 (81-176 *rpsL*^{Sm} Δ*astA fliF*$_{\Delta A176\text{-}S176}$), JMB2157 (81-176 *rpsL*^{Sm} Δ*astA fliG*$_{\Delta N'10AA}$), DAR125 (81-176 *rpsL*^{Sm} Δ*astA* Δ*fliQ*), SNJ1046 (81-176 *rpsL*^{Sm} Δ*astA* Δ*flgB*), and SNJ1048 (81-176 *rpsL*^{Sm} Δ*astA* Δ*flgC*).

Creation of *C. jejuni* 81-176 *rpsL*^{Sm} Δ*astA* double mutants lacking *flhA* and *fliF* or *fliG* was achieved by electroporating DRH979 (81-176 *rpsL*^{Sm} Δ*astA* Δ*flhA*) with pDRH2534 or pALU115. Transformants were selected on MH agar containing chloramphenicol and verified by colony PCR to recover JMB1771 (81-176 *rpsL*^{Sm} Δ*astA* Δ*flhA fliF*::*cat-rpsL*) and JMB1774 (81-176 *rpsL*^{Sm} Δ*astA* Δ*flhA fliF*::*cat-rpsL*). JMB1771 was then electroporated with pJMB1204 and JMB1774 was electroporated with pDRH2073. Transformants were recovered on

streptomycin and verified by colony PCR to isolate JMB1815 (81-176 *rpsL*^{Sm} Δ*astA* Δ*fliG* Δ*flhA*) and JMB1821 (81-176 *rpsL*^{Sm} Δ*astA* Δ*fliF* Δ*flhA*).

Previously constructed *C. jejuni rpsL*^{Sm} Δ*astA* mutant strains containing the *flaB*::*astA* transcriptional reporter are listed in Table S1. Additional C. *jejuni rpsL*^{Sm} Δ*astA* strains containing *flaB*::*astA* were created by electorporating pDRH665 into DRH2077, DRH2468, JMB1230, JMB1242, JMB1407, JMB1415, JMB1517, JMB1641, JMB1748, JMB1756, JMB1960, JMB2004, JMB2008, JMB2157, SNJ915, SNJ925, SNJ1046, SNJ1048, and DAR125 (4). Transformants were recovered on MH agar with kanamycin and verified by colony PCR.

Construction of plasmids for *in trans* complementation analysis. Primers were designed for *C. jejuni* 81-176 *fliF* so that either the wild-type gene, *fliF* mutants that encode deletions of the N- or C-terminus or point mutations would be amplified. Additionally, $fliF_{\Delta G511\text{-}S520}$ was amplified from pJMB1516. All primers used for PCR contained 5' BamHI or PstI sites to facilitate cloning into appropriate plasmids. PCR-amplified DNA fragments were then cloned into BamHI-digested pECO102 or BamHI- and PstI-digested pECO102 to generate pDRH2309, pJMB1505, pJMB1518, pJMB1519, pJMB1560, pJMB1561, and pJMB1609. These plasmids allowed for the constitutive expression of WT and mutant *fliF* genes from the *cat* promoter in pECO102. All plasmids were verified by DNA sequencing.

Primers were designed for *C. jejuni* 81-176 *fliG* to amplify the coding sequence from codon 2 through the stop codon with in-frame 5' BamHI sites. After PCR and digestion with BamHI, the fragment was cloned into BamHI-digested pCE107 to generate pJMB1842. Primers were then designed with 5' NotI or XhoI sites to amplify a region of pJMB1842 containing the promoter of *flaA* fused to the *fliG* coding sequence. These primers were also used in PCR with pCE107 to amplify the *flaA* promoter only. The resulting DNA fragments were digested and

then cloned into NotI- and XhoI-digested pRY112 to generate pJMB2074 (pRY112::p*flaA*) and pJMB2071 (pRY112::p*flaA-fliG*).

All plasmids including pECO102 were transferred first into DH5α/pRK212.1 before conjugation into either DRH2077, DRH2113, JMB1242, or JMB1258. Transconjugants were selected for growth on MH agar containing streptomycin, trimethoprim, and either kanamycin or chloramphenicol.

Construction of plasmids and strains for expression of FLAG-tagged proteins. pDAR965 was created for ease in cloning genes downstream of and in-frame with the *cat* promoter containing a start codon and N-terminal FLAG tag. To construct pDAR965, the *cat* promoter was amplified from pRY109 with primers to result in a DNA fragment containing a 5' XbaI site upstream of the *cat* promoter and a FLAG-tag encoded immediately after the start codon with an in-frame BamHI site downstream. This fragment was then digested with XbaI and BamHI and cloned into XbaI- and BamHI-digested pRY112 to result in pDAR965.

Primers were designed to amplify from codon 2 to the stop codon wild-type *fliF* from *C. jejuni* 81-176 chromosomal DNA or $fliF_{\Delta A175\text{-}S176}$ from pJMB1827. These primers also contained in-frame BamHI or PstI sites to the 5' ends of the amplified constructs. The wild-type *fliF* DNA fragment was digested with BamHI and then ligated into BamHI-digested pDAR965 to create pJMB2032. $fliF_{\Delta A175\text{-}S176}$ was digested with BamHI and PstI and then ligated into BamHI- and PstI-digested pDAR965 to result in pJMB2212. These plasmids expressed N-terminal FLAG-tagged FliF proteins from the *cat* promoter.

Primers were designed with in-frame BamHI sites to amplify *fliG* from the chromosome of *C. jejuni* 81-176 from codon 2 through the stop codon. In addition, one primer also encoded a FLAG-tag between the last codon and the stop codon. The DNA fragment was then digested

with BamHI and ligated into BamHI-digested pCE107 to create pJMB1746. All plasmids were verified by DNA sequencing.

All plasmids including pDAR965 and pCE107 were transferred first into DH5α/pRK212.1 before conjugation into either DRH2077, JMB1242, JMB1821, or JMB1815. Transconjugants were selected for growth on MH agar containing streptomycin, trimethoprim, and chloramphenicol or kanamycin.

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