Characterization of *Campylobacter jejuni* RacRS reveals a role in the heat shock response, motility, and maintenance of cell length population homogeneity

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ABSTRACT

Campylobacter jejuni commensally colonizes the cecum of birds. The RacR (reduced ability to colonize) response regulator was previously shown to be important in avian colonization. To explore means by which RacR and its cognate sensor kinase RacS may modulate C. jejuni physiology and colonization, ΔracR and ΔracS mutants were constructed in the invasive, virulent strain 81-176, and extensive phenotypic analyses were undertaken. Both ΔracR and ΔracS mutants exhibited a ~100-fold defect in chick colonization despite no (ΔracS) or minimal (ΔracR) growth defects at 42°C, the avian body temperature. Each mutant was defective for colony formation at 44°C and in the presence of 0.8% NaCl, each of which are stresses associated with the heat shock response. Promoter-reporter and RT-qPCR analyses revealed that RacR activates racRS and represses dnaJ. Although disregulation of several other heat shock genes was not observed at 38°C, ΔracR and ΔracS mutants exhibited a diminished upregulation of these genes upon rapid temperature upshift. Furthermore, ΔracR and ΔracS mutants displayed increased length heterogeneity during exponential growth, with a high proportion of filamented bacteria. Filamented bacteria had reduced swimming speed and were defective for invasion of Caco-2 epithelial cells. Soft agar studies also revealed that loss of racR or racS resulted in whole-population motility defects through viscous media. These findings reveal new roles for RacRS in C. jejuni physiology, each of which is likely important during colonization of the avian host.
INTRODUCTION

_Campylobacter jejuni_ (C. jejuni) is a Gram-negative, highly motile, microaerophilic, capnophilic, and modestly thermophilic bacterium. _C. jejuni_ is the leading cause of bacterial diarrheal disease in the developed world, accounting for more infections than _E. coli_ O157:H7, _Salmonella_ spp., and _Shigella_ spp. combined (3, 4, 10). Although ingestion of contaminated drinking water and milk are concerns, it is believed that 50-70% of _C. jejuni_ infections result from the consumption of poultry and related products (3). Acute campylobacteriosis presents as stomach cramps, fever, and severe, often bloody, diarrhea (3, 15). Although gastroenteritis associated with _C. jejuni_ is generally self-limiting, bacteremia and other complications can occur in immunocompromised individuals. Infections can also lead to more serious medical sequelae including Guillain-Barré syndrome, a demyelinating polyneuropathy causing bilateral paralysis (2). Furthermore, the emergence of antibiotic resistant _C. jejuni_ isolates has accelerated in recent years (14). Despite its prevalence as a human pathogen, many aspects of _C. jejuni_ physiology are poorly understood.

Although a pathogen in humans, _C. jejuni_ is able to colonize the gastrointestinal tract of birds in an asymptomatic manner. The optimal growth temperature of _C. jejuni_ in the laboratory is 37-42°C, correlating with the body temperatures of humans (37°C) and birds (42°C). Once colonization occurs, the bacteria can quickly spread through a chicken flock (56). The primary site of colonization is the deep crypts of the cecum (6), and in poultry _C. jejuni_ can propagate to 10^10 colony forming units (CFU) per gram of intestinal tissue (77). Chemotaxis and motility are required to reach this privileged niche (28, 47). Relative to other enteric Gram-negative pathogens, _C. jejuni_ has a significantly higher swimming speed which increases further with elevated viscosity (22, 55), and it is likely that this adaptation allows the organisms to traverse the viscous mucus layer that covers the intestinal epithelium. Although the number of transcriptional regulatory genes in _C. jejuni_ is limited, partly owing to its small genome size (48), a relatively high proportion of _C. jejuni_ regulatory elements are devoted to synthesis of its bipolar flagella, motility and chemotaxis (32, 41). Other factors such as its short corkscrew morphology are also predicted to play an important role in motility of _C. jejuni_ (76).
The ability of a bacterium to counter environmental insults is dependent on its genetic repertoire. As bacteria sense environmental changes that challenge their intracellular homeostasis, they must carefully modulate the expression of specific genes and pathways to alleviate stress. Regulation of this can be performed by bacterial two-component systems (59). These systems typically consist of a membrane-anchored sensor kinase (SK) and a cognate response regulator (RR). The canonical SK commonly has both kinase and phosphatase activity on its cognate response regulator, which translates into a dynamic and continuous response to extracellular stimuli (71). The phosphate on the histidine of the SK is transferred onto an aspartate residue on the RR, which in many cases is a transcription factor. The phosphorylated RR acquires high affinity for DNA and binds at specific regulatory sequences to activate or repress gene transcription (58, 59).

The *C. jejuni* genome is predicted to encode 11 response regulators, 6 sensor kinases, and one hybrid sensor response regulator protein (48). To date, mutants in genes encoding the response regulators *cbrR, cheY, flgR, racR* and *dccR* as well as the sensor kinase *cprS* have all been shown to have defects in colonization or the ability to cause disease in an animal model (12, 50, 64, 70, 73). Of these, the Δ*cheY* and Δ*flgR* mutants were non-motile, whereas the Δ*cprS* mutant was modestly hypermotile. RacRS was one of the first two-component systems identified in *C. jejuni*. RacR was previously shown to be important for avian colonization and growth at 42°C, the body temperature of chickens (12). Proteomic analysis indicated that RacR influences expression of up to 11 proteins in both a temperature-dependent and -independent manner (12). Genome analysis has revealed that the RacRS two-component system is conserved among multiple *Campylobacter* spp. (24), and racR was detected in 98% of *C. jejuni* clinical isolates (18). The *racRS* operon was disregulated in several *in vitro* conditions similar to the environments encountered by *C. jejuni* during infection (E.C.G., unpublished observations). Notably, some of these conditions were independent of the switch between 37°C and 42°C, suggesting that the system likely plays multiple roles in *C. jejuni* physiology, colonization and pathogenesis.
In the present study, we provide evidence that RacR and RacS enable *C. jejuni* to overcome stresses associated with the heat shock response. We also reveal the importance of this two-component system in maintaining bacterial motility and cell length population homogeneity.
MATERIALS AND METHODS

Bacterial Strains and Growth Conditions.

Studies were performed primarily using the *C. jejuni* parent strain 81-176, but also 81116 and 11168 where indicated. *C. jejuni* derived strains are listed in Table 1. *C. jejuni* was cultured in Mueller-Hinton broth (MH; Oxoid, Hampshire, England) or on agar plates. *C. jejuni* growth media was supplemented with 10 µg ml⁻¹ vancomycin and 5 µg ml⁻¹ trimethoprim (MH-TV). When appropriate, bacterial growth media were supplemented with kanamycin and/or chloramphenicol (Sigma, Oakville, ON) at 50 or 20 µg ml⁻¹, respectively. *C. jejuni* was routinely cultured in a 12% CO₂/6% O₂ atmosphere, using either a Sanyo tri-gas incubator (plates) or an Oxoid CampyGen system (broth cultures). Unless otherwise noted, standard *C. jejuni* culture temperature was 38°C. DNA manipulations were performed in an *E. coli* DH5α background strain which was routinely cultured at 37°C in Luria-Bertani (Sigma) with 100 µg ml⁻¹ ampicillin, 50 µg ml⁻¹ kanamycin, or 30 µg ml⁻¹ chloramphenicol when appropriate.

Recombinant DNA techniques.

Recombinant DNA manipulation was performed as described in Sambrook and Russell (52). DNA modifying enzymes were purchased from New England Biolabs (Mississauga, ON) and Invitrogen (Burlington, ON). Plasmids were isolated from bacteria utilizing the Qiagen Qiaprep Spin miniprep kit (Qiagen, Mississauga, ON).

Construction of ΔracR, ΔracS, ΔdnaJ, ΔracR/ΔdnaJ mutants and the ΔracR<sup>C</sup> complemented strain.

The ΔracR mutant was constructed by PCR amplification of racR from 81-176 genomic DNA using the primers racR-1 and racR-2 and cloning the PCR product into a commercial vector pGEM-T (Promega) (see Table S1 for primers used in this study). Inverse PCR was then performed using primers racR-3 and racR-4. The resulting amplicon and the plasmid pUC18K-2, carrying a nonpolar kanamycin resistance (*aphA-3*) cassette (26), were each digested with *BamHI* and *EcoRI* restriction enzymes and ligated to form the plasmid pGEM-racR-KO. The plasmid was introduced into *C. jejuni* 81-176, 11168 or 81116 by natural transformation. Kanamycin resistant colonies were isolated and ΔracR mutants were confirmed via PCR and sequencing analysis.
The \( \Delta \text{racS} \) mutant was constructed in a similar manner as \( \Delta \text{racR} \) except the initial primers were \text{racS-1} \text{ and racS-2}, and inverse PCR was performed using the primers racS-3 and racS-4.

The \( \Delta \text{dnaJ} \) mutant was constructed in a similar manner as \( \Delta \text{racR} \) except the initial primers were \text{dnaJ-1} \text{ and dnaJ-2} \text{ and inverse PCR was performed using primers dnaJ-3 and dnaJ-4}. The resulting amplicon and plasmid pRY109 carrying a chloramphenicol resistance \((\text{cat})\) cassette (72) were digested with \text{EcoRI} \text{ and ligated to form plasmid pGEM-dnaJ-KO}. The \text{cat} \text{ cassette was confirmed to be in the same orientation as the dnaJ ORF}. The plasmid was introduced into \( C. \text{jejuni} \) 81-176 \text{ wt or 81-176 } \Delta \text{racR} \text{ by natural transformation to create strains } \Delta \text{dnaJ} \text{ and } \Delta \text{racR/dnaJ}, \text{ respectively.}

To generate a \( \Delta \text{racR} \) complemented strain \((\Delta \text{racR}^C)\), the \text{racR ORF} \text{ and 323 bp of upstream DNA were PCR amplified from 81-176 genomic DNA using the primers 5'-racR-Spel and 3'-racR-EcoRI}. \text{ The amplicon was digested with Spel and EcoRI and ligated to plasmid pRRC (34), which was digested with MfeI and XbaI to generate compatible DNA overhangs}. \text{ The E. coli derived plasmid was introduced by natural transformation into the \( \Delta \text{racR} \) mutant. Chloramphenicol and kanamycin resistant colonies were isolated and the presence of the complementing DNA at a ribosomal RNA spacer region as well as retention of the \text{racR} \text{ deletion at the native locus was confirmed by PCR.}

**Physiological studies of \( \Delta \text{racR}, \Delta \text{racS}, \Delta \text{dnaJ} \text{ and } \Delta \text{dnaJ/racR} \text{ and } \Delta \text{racR}^C.**

Growth curves were performed in MH-TV broth from an overnight \( C. \text{jejuni} \) cultures diluted to an initial OD\textsubscript{600} of 0.05, and the optical density was measured at various time points specific to the experiment. To investigate \( C. \text{jejuni} \) colony formation at various temperatures, broth cultures were normalized to an OD\textsubscript{600} of 0.1, and ten-fold serial dilutions were spot-plated on solid media and incubated at 38°C, 42°C, 44°C, 46°C, 38°C+0.8% NaCl and 42°C+0.8% NaCl for 48 hours at which time colony forming units (CFU) were enumerated.

**Chick colonization assays.**

The colonization assays were performed essentially as described (28). Briefly, day-of-hatch chicks (white-Leghorn, Charles River Laboratories) were orally inoculated with ~10\textsuperscript{4} CFU from an overnight broth culture diluted in PBS. After 6 days, birds were sacrificed and the ceca were removed.
Ceca were weighed, homogenized, serially diluted, and plated on MH agar containing 30 µg ml⁻¹ cefoperazone and 10 µg ml⁻¹ trimethoprim. *C. jejuni* colonies were enumerated, and the CFU g⁻¹ cecal matter recorded.

**Construction and utilization of a luciferase reporter vector.**

To construct a luciferase promoter reporter system in *C. jejuni*, the *luxCDABE* operon of *Photorhabdus luminescens* was PCR amplified from vector pCS26 (9) using primers 5'-SmaI-luxC and 3'-PstI-luxE. The amplicon was digested with SmaI and PstI and subsequently ligated to the similarly digested plasmid pRY112 (72). The resulting vector, pRY112-*luxCDABE*, can be used to swap the transcriptional regulatory sequence upstream of the *luxCDABE* cassette encoding luciferase biosynthetic enzymes. The *racR* and *dnaJ* regulatory sequences were PCR amplified from 81-176 genomic DNA and subcloned upstream of the *lux* operon to generate *PracR-*luxCDABE pRY112 and *PdnaJ-*luxCDABE pRY112 (Table S2). Through triparental mating with *E. coli*, performed as previously described (44), the chloramphenicol resistance plasmids were introduced into *C. jejuni* strain DRH461, a streptomycin resistant 81-176 derivative (29). Once luminescent colonies of strain DRH461 were isolated, the plasmids were purified and transformed into 81-176 or Δ*racR* and Δ*racS* mutant derivatives.

Growth curves were performed at 38°C as described above, and measurements of light production were carried out at 0, 3, 6, 9 and 12 hours post-inoculation during which 100µL of culture was removed and optical density and luminescence measurements were performed using a Varioskan Flash Luminometer (Thermo Scientific).

To investigate if RacR directly binds the *racR-dnaJ* intergenic region, a *dnaJ* promoter-luciferase fusion was assessed in *E. coli* also expressing recombinant RacR. To construct the expression plasmid, the *racR* ORF was amplified using primers 5'-racR-EcoRI and 3'-racR-PstI and ligated into pBAD24 (27) to form pBAD24-*racR*. Using primers 5'-pBAD-Xhol and 3'-pBAD-Apal, *araC* or *racR-araC* were PCR amplified from pBAD24 or pBAD24-*racR*, respectively. The amplicons were digested with Xhol and Apal and ligated to *PdnaJ-*luxCDABE pRY112 to form *PdnaJ-*luxCDABE/araC and *PdnaJ-*
luxCDABE/araC/PBAD-racR pRY112. The generated vectors were transformed into E. coli strain BW27783 (37). Luciferase expression experiments were performed by growing an E. coli culture to early log phase in LB medium supplemented with 30 µg mL−1 chloramphenicol and inducing racR expression by the addition of 0.02% arabinose. Luciferase dependent light activity was measured 6 hours after induction as described above.

Real-time reverse transcription quantitative PCR.

C. jejuni were grown to early log phase (OD_{600} ~0.15) at 38°C under microaerophilic conditions and shifted to 44°C for 15 minutes. A control culture was grown in parallel and remained at 38°C. RNA isolation and cDNA synthesis were carried out as previously described (64). The qPCR reactions were performed for dnaJ, dnaK, and groEL by using designed primers (Table S1). The expression of rpoA was used as an internal control as this gene has previously been shown to be invariant during temperature upshift (57). Reactions were set up with IQ SYBR Green Supermix (Bio-Rad) and performed on a CFX96 Real-Time PCR Detection System (Bio-Rad). Expression differences were calculated using the ∆∆CT method.

Bright field microscopy.

For bright field microscopy of log-phase bacteria, C. jejuni were harvested from MH-TV broth cultures (OD_{600} of 0.25-0.35) 6 hours after inoculation from an overnight culture, and samples were immediately spotted onto glass slides overlaid with 1% agarose pads. For tracking of swimming bacteria, C. jejuni were harvested at the same density, diluted 1/10 in prewarmed MH-TV broth, and spotted onto glass microscope slides. For tracking of swimming speed, 50 individual bacteria were selected from each strain and in the case of ∆racR, represented the observed length variation among the mutant population. Images were captured with a Nikon Eclipse TE2000-U microscope equipped with 40x and 100x objectives and a Hamamatsu ORCA camera system. Micrograph analyses were performed using NIS-Elements AR Imaging Software (Melville, NY) and ImageJ (NIH, USA).

Soft agar motility assays.
C. jejuni broth cultures were propagated for 12-16 hours in MH-TV broth. Cultures were diluted to an OD₆₀₀ of 0.02 and 2.5 µL of the suspension was stab-inoculated into plates containing MH-TV medium and 0.4%, 0.5% or 0.6% agar. When coinoculation of \( wt \) and \( ΔracR \) was performed, the bacterial culture of each strain was normalized to OD₆₀₀ of 0.02, mixed at a 1:1 ratio, and 2.5 µL of culture was stab-inoculated into the motility plate. Plates were incubated at 38°C for 28 hours, at which time motility was quantified by measuring the diameter of the migration front. When microscopic examination of cells in the motility plate was performed, a sterile toothpick was used to harvest bacteria from specific locations of the semi-solid motility halo and light microscopy was carried out as described. To quantify the CFU ratios of \( wt \) and \( ΔracR \) mutants in coinoculated motility plates, bacteria were harvested from the semi-solid agar with a sterile toothpick, serially diluted 10-fold and plated on MH and MH with kanamycin.

Construction of GFP expressing strains, tissue culture infections, and immunofluorescent analysis.

The \( gfp \) ORF was amplified from plasmid pFPV25 (17) using primers 5'-SmaI-gfp and 3'-XhoI-gfp and subcloned into plasmid pRY112. The transcriptional regulatory sequence of the \( atpF' \) ORF (CJJ81176_0137) was amplified with primer pairs 5'-NotI-atpF' and 3'-SmaI-atpF' from strain 81-176 and subcloned upstream of the \( gfp \) ORF to generate plasmids \( PatpF'-gfp \) pRY112 (Table S2). The plasmid was introduced into \( C. jejuni \) 81-176 as described above.

Tissue culture infections were performed with Caco-2 intestinal epithelial cells with a \( C. jejuni \) multiplicity of infection (MOI) of ~25 (2.5x10⁶ bacteria per well) essentially as described (26). Briefly, 10⁵ Caco-2 cells were seeded on glass cover slips in 24-well plates and grown overnight. Overnight log-phase cultures of \( C. jejuni \) harboring \( PatpF'-gfp \) were washed twice with MEM and resuspended at an OD₆₀₀ of 0.0005. One milliliter of bacterial suspension was added per well, and Caco-2/\( C. jejuni \) were incubated for 3 hours. Samples were washed twice in MEM, and cell-associated bacteria were fixed in 4% formalin solution in PBS. Samples were permeabilized for 15 min in PBS with 0.2% Triton X-100 when appropriate. Immunostaining was performed by treating the fixed samples with rabbit anti-Campylobacter antibodies.
(1:200, US Biological) and Alexa 568 (red) conjugated goat anti-rabbit secondary antibody (1:500, Molecular Probes). Samples were mounted onto glass slides with ProLong Gold antifade reagent with DAPI (Invitrogen). Fluorescent images were captured with an Olympus FluoView FV1000 Confocal Laser Scanning Microscope (Olympus) equipped with appropriate fluorescent filters. The experiment was performed three times for each strain, during which five individual fields containing 25-50 bacteria per image were acquired.

Statistical Analysis.

Data obtained from microscopic tracking and halo diameters in motility assays and luciferase expression experiments were analyzed using the Student’s two-tailed t-test. Statistical analysis of the data from the chick infection experiment was performed using the two-sided Mann-Whitney U-test. p-values are indicated when appropriate.
C. jejuni strain 81-176 ΔracR and ΔracS mutants are defective in chick colonization but display only modest (ΔracR) to no (ΔracS) growth defects at 42°C.

The ΔracR mutant of strain 81116 was reported to have a slower in vitro growth rate at 42°C and to be significantly defective in chick colonization (12). We wanted to assess the role of racR/S in in vitro growth as well as chick colonization in the highly invasive C. jejuni strain 81-176. Neither ΔracR nor ΔracS were defective for growth at 38°C (Fig 1A). At the avian body temperature of 42°C, the ΔracR mutant reached a modestly lower final optical density in broth culture than wild type (wt), although the growth rate in early stages was unaffected (Fig 1B). The ΔracS mutant exhibited no defects in either growth rate or final optical density at 42°C.

To establish a requirement for RacRS in avian colonization for strain 81-176, we tested the ΔracR and ΔracS mutants in a one-day-old chick colonization model. 10^4 CFU were orally administered to day-of-hatch Leghorn chicks, and cecal colonization was enumerated 6 days post-infection. Both the ΔracR and ΔracS mutants were recovered at approximately 100-fold fewer CFU g^-1 of cecal tissue relative to 81-176 wt (Fig 1C). The ΔracR colonization data are consistent with the findings of Bras et al. demonstrating the importance of racR for chicken colonization (12). These data also identify a role for racS in colonization despite having no apparent growth defect in vitro at 42°C, and suggest that RacRS likely influences physiological properties other than the ability to grow at 42°C that are also important for colonization.

RacR activates racRS and represses dnaJ.

To identify potential factors contributing to the diminished in vivo fitness of ΔracR and ΔracS mutants, we first investigated whether expression of racRS and/or dnaJ was disregulated in the mutant strains. We tested racRS because transcription factors often regulate their own expression, and dnaJ, encoding a heat shock chaperone previously shown to be important for chick colonization (39), because it
is divergently transcribed from racRS with 157 base pairs separating the two operons. For studies in C. jejuni, we utilized a luciferase promoter-reporter fusion which enables the measurement of gene expression in real-time at different points of the bacterial growth curve (1, 35). Promoter-luciferase fusions ($P_{racR}$-luxCDABE and $P_{dnaJ}$-luxCDABE) were constructed in the C. jejuni replicating vector pRY112 and introduced to C. jejuni wt, ΔracR and ΔracS strains as described in Methods. The $P_{racR}$-luxCDABE reporter had a lower level of expression in both mutants at 38°C relative to the wt parent strain (Fig 2A). Conversely, the $P_{dnaJ}$-luxCDABE fusion was expressed approximately 3.3-fold higher at 38°C in the ΔracR mutant relative to wt, with ΔracS exhibiting an intermediate level of expression (Fig 2B). Similar trends were observed at 42°C, with analysis of dnaJ expression by RT-qPCR also showing a ~2-fold increase in ΔracR compared to wt (data not shown). Several other heat shock gene operons (groESL, grpE/dnaK, clpB) and hspR (encoding a repressor of those operons) were assessed by promoter-luciferase fusions and RT-qPCR analyses, but no expression differences in ΔracR compared to wt at 38°C were observed (data not shown).

We next investigated whether RacR directly binds the intergenic region between racRS and dnaJ. Electrophoretic mobility shift assays (EMSA) were performed using the racRS-dnaJ intergenic DNA sequence and recombinant RacR purified from E. coli. However, we were unable to establish specific binding using this strategy, potentially due to lack of or unstable RacR phosphorylation, which has hindered EMSA approaches in other systems (59). As an alternative, we developed a synthetic system to study C. jejuni transcriptional regulation in E. coli. It was previously found that E. coli RNA polymerase can recognize C. jejuni promoters and initiate transcription of downstream genes (69). As repression frequently involves direct regulator-DNA binding whereas activation can be more complicated (i.e., requiring additional variables such as regulator interaction with RNA polymerase), we used negative regulation of dnaJ as a readout for direct interaction of RacR with the racRS-dnaJ intergenic region. araC and racR were cloned under the control of constitutive ($P_c$) and arabinose-inducible ($P_{bad}$) promoters, respectively, into the pRY112 plasmid described above harbouring the $P_{dnaJ}$-luxCDABE.
luciferase-reporter fusion (Fig 2C). The addition of arabinose to *E. coli* harbouring this plasmid will induce *racR* expression and RacR protein production, allowing determination of whether transcription of the exogenous promoter-luciferase fusion is directly regulated by RacR. A control plasmid harbouring all components except *P_{Bad-racR}* were also constructed to ensure that any significant differences seen +/- arabinose were due to the presence or absence of RacR. Upon arabinose induction, light produced by *E. coli* containing *P_{dnaJ-luxCDABE/araC/P_{Bad-racR}}* decreased approximately 2-fold compared to uninduced samples (*p*<0.0001) (Fig 2D). *E. coli* harbouring the control plasmid (*P_{dnaJ-luxCDABE/araC}* did not exhibit statistically significant changes in light production following arabinose induction. This suggests that RacR interacts with the DNA region between the *dnaJ* and *racRS* operons to modulate their expression.

Δ*racR* and Δ*racS* mutants are sensitive to elevated temperatures and hyperosmotic stress.

Heat shock proteins (HSPs) have an important function in allowing organisms to overcome physiologically stressful conditions such as elevated temperatures and osmotic stress. As RacR regulates *dnaJ* expression, we first investigated survival and colony formation by Δ*racR* and Δ*racS* at above-optimal temperatures. Serial dilutions of log-phase *C. jejuni* 81-176 wt and mutant strains were inoculated on solid media and incubated at 38°C, 42°C, 44°C and 46°C. While Δ*racR* and Δ*racS* strains did not yield defects in colony formation at 38 or 42°C, there was a significant defect in colony formation for both mutants when propagated at 44°C (Fig 3A). Complementation of *racR* in trans (Δ*racR*C) partially rescued this defect. At 46°C, all strains including *wt* failed to form colonies after 48 hours (data not shown).

Osmotolerance is also influenced by HSPs in numerous bacterial species (7), and *C. jejuni* is likely to encounter hyperosmotic conditions during gastrointestinal colonization of vertebrate hosts (23, 38). On plates supplemented with 0.8% NaCl, Δ*racR* was modestly defective for growth and colony formation at 38°C and severely defective at 42°C, with Δ*racS* exhibiting a milder phenotype (Fig 3B).
Complementation of racR restored osmotolerance to wt levels. These findings indicate that RacRS provides resistance during temperature and osmotic stress and identify an additive detrimental effect on ΔracR and ΔracS mutant survival when the stressors are present simultaneously.

DNAJ disregulation is not the sole contributing factor to ΔracR and ΔracS temperature sensitivity, with other heat shock genes also exhibiting dampened induction upon temperature upshift.

dnaJ is not essential for C. jejuni under non-stressful laboratory conditions but has a role in temperature resistance (39). We next tested if the temperature sensitivity of ΔracR was caused by disregulation of dnaJ in the mutant strain. As no system currently exists for the inducible expression/repression of genes in C. jejuni, we generated a ΔdnaJ/ΔracR double mutant to investigate whether disregulation of dnaJ might account for the observed ΔracR defects. Neither ΔdnaJ nor a ΔdnaJ/ΔracR double mutant was defective in colony formation at 42°C, although the ΔdnaJ/ΔracR mutant formed smaller and fewer colonies than wt or either of the single mutants. At 44°C, the ΔdnaJ mutant had a substantial defect in colony formation which was more severe than the ΔracR mutant, and the ΔdnaJ/ΔracR double mutant was unable to form colonies after 48 hour incubation (Fig 4A). These data indicate that dnaJ disregulation is not the sole factor contributing to the temperature sensitivity of ΔracR.

C. jejuni exhibits a transient increase in expression of multiple HSPs upon temperature upshift (57). To expand our understanding of potential mechanisms underlying temperature sensitivity of ΔracR and ΔracS, we tested whether this response might be generally defective in our mutant strains. C. jejuni were grown to early log phase at 38°C, then shifted to 44°C for 15 minutes or allowed to remain at 38°C. Expression of dnaJ, dnaK and groEL at each temperature was quantified by RT-qPCR. Data are presented as the fold induction at 44°C relative to 38°C of each gene in wt, ΔracR, and ΔracS strains. As expected, expression of all three HSP genes was induced in wt following temperature upshift (Fig 4B). In the ΔracR and ΔracS mutants, induction at 44°C was negligible for dnaJ (Fig. 4B), as might be predicted given
RacR’s likely direct effect on dnaJ expression (Fig. 2). However, induction at 44°C was also significantly dampened for dnaK and groEL (Fig 4B), neither of which were affected in ΔracR at 38°C (data not shown). For instance, while dnaK expression increased 5.2-fold in wt C. jejuni, the increase was only 2- and 2.5-fold in the ΔracR and ΔracS mutants, respectively. A similar trend was observed for groEL. Thus, in addition to directly regulating dnaJ, RacRS is also required for proper up-regulation of other HSP genes under heat shock conditions (Fig. 4B).

ΔracR and ΔracS mutants have increased cell length heterogeneity resulting from filamented bacteria in the population.

HSP disregulation has been linked to altered bacterial length in C. jejuni and other bacterial species (5, 11, 43, 63). To determine if loss of racR or racS affects cell length, we quantified the length of individual bacteria in the population using light micrographs and imaging software (Fig 5A-C and Table 2). While the wt population was primarily composed of short cells, the ΔracR mutant exhibited an increased mean cell length relative to wt, with the population distributed broadly over multiple lengths. For instance, ~63% of the wt population was less than 1.5 µm in length, while only ~38% of the ΔracR mutant was less than 1.5 µm (Fig 5C and Table 2). The degree of cell length heterogeneity within the wt, ΔracR, and ΔracS populations was quantified by calculating the coefficient of variation (Cv), which is a ratio of the standard deviation to the mean and, in our case, provides a normalized measure of the population length distribution when comparing strains with different mean cell lengths. The Cv was two times higher for the ΔracR mutant compared to wt, further supporting cell length heterogeneity in the ΔracR mutant strain (Table 2). The ΔracR<sup>C</sup> complemented strain exhibited cell length and population distribution characteristics similar to wt (Table 2), while the ΔracS mutant exhibited phenotypes intermediate to those of wt and ΔracR (Fig 5C and Table 2).

Filamentation of ΔracR causes motility and epithelial cell invasion defects.
We next determined whether filamentation of ΔracR cells affected other attributes linked to C. jejuni colonization and pathogenesis. A previous report suggested that RacR was not required for C. jejuni motility (12); however, we hypothesized that elongated bacteria may exhibit motility defects because of their increased size. Time-lapse microscopy was used to assess the swimming speed of individual C. jejuni bacteria in broth cultures as a function of cell length in the wt and ΔracR populations (Fig. 6A). To normalize for Brownian motion, ethanol-treated bacteria were imaged and tracked to provide a baseline for subsequent analysis of swimming speed. In live cultures, only bacteria that were moving at a speed greater than 4 times that of ethanol-treated cultures were analyzed. The mean swimming speeds of wt and ΔracR C. jejuni were 28 ± 10 µm s⁻¹ and 24 ± 10 µm s⁻¹, respectively. Shorter ΔracR cells (<3.5 µm) had an average swimming rate of 29 ± 10 µm s⁻¹; however, ΔracR cells that were >3.5 µm in length had an average swimming speed of 15.0 ± 3.5 µm s⁻¹ (p<0.0001). In addition, short (<3.5 µm) ΔracR cells were capable of reaching speeds up to 55 µm s⁻¹ similar to wt, while ΔracR cells >3.5 µm in length did not exceed 25 µm/s. The only tracked wt cell >3.5 µm in length (3.9 µm) likewise swam at 22.6 µm s⁻¹. Filamented cells also changed direction more slowly than shorter cells (data not shown). Transmission electron microscopy indicated that the position and length of flagella were unaltered in ΔracR, and ΔracS mutants as well as among individual bacteria of different lengths in the ΔracR and ΔracS populations (data not shown). Single cell tracking also demonstrated that the elongated cells were viable, as their swimming speed was more than 4 times greater than that of ethanol treated bacteria in all but one examined individual bacterium (data not shown). Collectively, these findings demonstrate that while shorter cells in both the wt and ΔracR mutant populations swam at a variety of speeds and at similar average rates in broth media, elongated ΔracR cells swam significantly more slowly.

The ability of C. jejuni to invade epithelial cells in vitro is frequently used as a marker for virulence (16, 21). Using a gentamicin protection assay and enumeration of CFU recovery, we did not observe a general defect for adherence or invasion of Caco-2 (epithelial) tissue culture cells for the ΔracR or ΔracS mutants (data not shown). However, we were intrigued by the possibility that elongated bacteria...
may exhibit a defect in adherence and/or invasion of epithelial cells. To visualize cell-associated bacteria, we utilized a combination of GFP-expressing *C. jejuni* and immunofluorescence. Caco-2 cells were infected with *C. jejuni* for three hours, at which time bacteria in the media above the cells were removed, cells and cell-associated bacteria were washed, and all samples were fixed and processed for confocal microscopy. Within the media fraction, both wt and ΔracR *C. jejuni* were generally more elongated than when the same strains were propagated in MH broth. However, ΔracR mutants in the media fraction were highly variable in length, with some individuals substantially longer than the wt strain and measuring up to 30 µm in length (a representative example is shown in Figure 6B). The cell-associated bacteria were more homogeneous in length for both wt and ΔracR, and were always short (<3.5 µm) (Figure 6B). Consistent with CFU recovery-based experiments, there was no striking difference between wt and ΔracR with respect to the total number of cell-associated bacteria. Application of a *C. jejuni* antibody to unpermeabilized versus permeabilized cells also indicated that cell-associated bacteria of both strains were predominantly intracellular, as the red antibody signal seldom colocalized with the green GFP-expressing bacteria when epithelial membranes remained intact, but readily colocalized when Caco-2 cell membranes were permeabilized prior to staining. Together, these results suggest an advantage for short bacteria in the invasion of epithelial cells and demonstrate that elongated individuals in the ΔracR population are defective for cell invasion.

**The ΔracR and ΔracS mutant populations are defective in motility through semi-solid agar.**

The single-cell analyses showing motility defects for filamented bacteria led us to revisit whether RacRS might also be important for general motility in semi-solid agar media, a substance of higher viscosity than liquid broth. Strains were inoculated into 0.4% agar MH plates, grown at 38°C or 42°C, and halo diameters documented and quantified. Halo diameter differences between mutant and wt strains were similar at both temperatures, thus only 38°C is shown (Fig. 7). Both ΔracR and ΔracS were impaired for motility in soft agar, exhibiting halos 50% and 58% the diameter of wt, respectively (Fig 7A, B). As these findings are inconsistent with the previous report describing no motility defect for a *C. jejuni* 81116
ΔracR mutant through semi-solid media or by microscopy, and as discrepancies between C. jejuni strains are frequently observed (19, 25), we constructed ΔracR mutants in two other commonly utilized C. jejuni strains, 11168 and 81116, and assessed motility in soft agar relative to wt. Although there were significant differences between the halo diameters of the three wt isolates, the ΔracR mutants had similar motility defects irrespective of the parent strain (Fig 7C). These findings demonstrate a clear role for RacRS in motility through soft agar in at least three C. jejuni strains.

Short bacteria in the ΔracR mutant population are defective in motility through semi-solid agar.

Consistent with the broth motility data described above (Fig. 6A), we also found that filamented ΔracR bacteria were more defective for migration through semi-solid agar than short ΔracR bacteria, as assessed by microscopic examination of ΔracR cells taken from the center (c), middle (m), and outer (o) zones of a motility halo (Fig. 8A). However, we still needed to reconcile why the general motility of three independent ΔracR mutant strains consistently appeared defective compared to the parent strain in soft agar despite no apparent motility differences between wt and ΔracR short bacteria in broth medium (Fig 6A). Several hypotheses were considered. First, to test whether the soft agar motility defect might reflect decreased growth rate of ΔracR in soft agar, wt and the ΔracR strain were inoculated into semi-solid media with a range of agar concentrations (0.4% - 0.6%). As the largest halo diameter in this range occurs in 0.4% agar, halo diameters in higher concentration agar should become less dependent on swimming speed and more reflective of growth. Increasing the agar concentration reduced the relative halo size difference between wt and the ΔracR mutant (Fig 8B), suggesting that the ΔracR bacteria are not defective for growth in semi-solid media. We also investigated if elongated bacteria in the ΔracR mutant population might be impeding the outward migration of shorter cells. To test this, we co-inoculated wt and ΔracR mutants into a motility plate alongside singly inoculated wt and racR strains. If elongated bacteria in the ΔracR mutant population impeded the outward migration of shorter cells, then the motility halo of the wt + ΔracR co-inoculated culture should appear smaller than that of wt alone. The motility
halo of co-inoculated wt + ΔracR was similar to wt inoculated alone (Fig 8C), thus blocking of short cells by elongated bacteria was unlikely. These findings led us finally to hypothesize that short cells in the ΔracR mutant population have reduced motility through semi-solid agar despite having no obvious swimming defect in liquid broth. To test this, we sampled bacteria from the migration front of a motility plate which had been co-inoculated with a wt + ΔracR, and enumerated CFU on MH agar (allows growth of both strains) or MH agar supplemented with kanamycin (selective for the ΔracR mutant). The recovered CFU of wt outnumbered ΔracR by a factor of 10^3-10^5 (Fig 8D), suggesting that wt bacteria were able to swim faster than the short ΔracR mutant cells. Together, these data indicate that while filamented bacteria swim more slowly than short bacteria, the short ΔracR bacteria and indeed the entire ΔracR population are defective in motility through semi-solid media.
DISCUSSION

*C. jejuni* is a significant burden to human health. In developed countries, where infection is believed to be primarily the result of consumption of contaminated poultry products, our understanding of bacterial colonization of chickens is paramount to the implementation of control strategies. Within poultry, *C. jejuni* is found at high numbers in the cecum and large intestine (6, 28), and localized within the deep crypts in the mucus layer in close proximity to epithelial cells (40). To reach its preferred niche, *C. jejuni* encounters stresses (e.g., osmotic, oxidative, acid) that pose survival challenges. The bacteria must also be able to migrate (swim) to an area of the gastrointestinal tract more suitable for growth and replication. *C. jejuni* likely utilizes highly evolved strategies to overcome these potential stressors, reach its preferred niche, and successfully colonize the avian host.

*C. jejuni* has a relatively limited number of two-component systems (48). Although genes encoding the response regulators CbrR, CheY, FlgR, RacR and DccR and the sensor kinase CprS are each important for animal colonization (12, 50, 64, 70, 73), the unique phenotypic profile of each mutant indicates a lack of functional redundancy between individual two-component systems. In this work, we have also established the importance of *racS* in colonization, and found that both *ΔracR* and *ΔracS* mutants in strain 81-176 exhibit a ~100-fold defect in colonizing one-day-old chicks. Our *ΔracR* chick data are consistent with the *ΔracR* colonization defect noted in a previous study (12); however, some discrepancies were observed regarding the fitness of *ΔracR* under non-stressful laboratory conditions. These results are puzzling but may stem from the fact that the previous experiments were performed in an 81116 strain background, whereas this study primarily utilized the highly invasive 81-176 strain. Nonetheless, it is now evident that *C. jejuni* requires not only RacR but also RacS for optimal chick colonization.

Given the modest (*ΔracR*) or nonexistent (*ΔracS*) growth defects at 42° C, we reasoned that other factor(s) important for colonization may be influenced by RacRS. Our observation of severe growth defects for both mutants at a true temperature stress of 44° C suggested a potential defect in induction of...
the heat shock response. This response is modulated by HSPs, which are of two general classes:
molecular chaperones that facilitate protein folding and ATP-dependent proteases that degrade misfolded polypeptides (20, 42, 67). HSPs are especially important during sudden exposure to stressful conditions, where they act post-translationally to prevent protein misfolding, denaturation and aggregation (61, 63, 67). The DnaK/DnaJ/GrpE complex plays a role in cell division, chromosome segregation, and resistance to extracellular stressors. (13, 61, 62). In \textit{C. jejuni}, \textit{dnaJ} is monocistronic while \textit{dnaK} and \textit{grpE} are co-transcribed. A \textit{C. jejuni} \textit{ΔdnaJ} mutant was unable to colonize chickens and was severely defective in colony formation at elevated temperatures (39). As \textit{dnaJ} is also divergently transcribed from \textit{racRS}, we hypothesized that RacR may directly regulate both its own operon and \textit{dnaJ}, and that \textit{dnaJ} disregulation might account for the \textit{ΔracR} and \textit{ΔracS} temperature sensitivity. Using RT-qPCR, promoter-lux reporters in \textit{C. jejuni}, and a new synthetic luciferase reporter system in \textit{E. coli}, we established that RacR activates \textit{racRS} and represses \textit{dnaJ}. However, analysis of a \textit{ΔracR/ΔdnaJ} double mutant indicated that \textit{dnaJ} disregulation may contribute to but is not exclusively responsible for the temperature sensitivity of a \textit{ΔracR} mutant at 44°C, suggesting a role for other factors as well. Unlike \textit{dnaJ}, other HSPs were not significantly disregulated in \textit{ΔracR} at 38°C and did not appear to be under direct RacR regulation. However, upregulation of other chaperone genes (\textit{dnaK} and \textit{groEL}) was dampened in the \textit{ΔracR} and \textit{ΔracS} mutants upon temperature upshift, suggesting a complex influence of RacRS on the heat shock response. The effect of RacRS on the heat shock response may also contribute to the \textit{ΔracR} growth defect in MH medium supplemented with 0.8% NaCl. Hyperosmotic conditions also cause accumulation of misfolded proteins, and in other bacteria, the heat shock response is required for survival. Of note, MH with added 0.8% NaCl more closely approximates the osmolarity of the chicken cecum than unsupplemented MH medium (23, 38), and we have recently found that osmotic stress rapidly induces HSP expression in \textit{C. jejuni} (A. Cameron and E.C. G., unpublished). Collectively, the stress survival attributes to which RacRS contributes and its pleiotropic effects on the heat shock response all likely contribute to fitness defects of the \textit{ΔracR} and \textit{ΔracS} mutants \textit{in vivo}. 

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Bacterial cell length is influenced by the timing of cell division, specifically the regulation and activity of the bacterial septation apparatus (74, 75). Disregulation of heat shock gene expression can affect this process, leading to an increase in cell length (11, 43, 60). For instance, C. jejuni mutants deleted for HspR, a transcriptional repressor of multiple heat shock genes, have increased cell length and elevated temperature sensitivity similar to that observed in ΔracR and ΔracS (5). We initially considered that filamentation in ΔracR and ΔracS may be the result of dnaJ overexpression; however, a ΔracR/ΔdnaJ mutant displayed the same population heterogeneity as ΔracR (data not shown). It is possible that very minor disregulation of other HSPs, undetectable by our analysis methods, may occur at 38°C and contribute to filamentation. As filamentation can also be caused by numerous other factors (i.e., DNA damage, nutritional limitation, etc.), this may be a highly complex phenomenon influenced by multiple other genes. Reasons underlying the variability in cell length associated with disruption of RacRS are also likely to be complex. A contributing factor may be the degree of synchronization of the bacterial culture. C. jejuni does not have a classic stationary phase typically found in many other Eubacteria (36), thus even carefully propagated and seemingly exponentially growing cultures may actually be composed of a diverse population of individuals in distinct growth phases. Our data suggest that RacRS may be important for ensuring proper timing of septation kinetics within a physiologically variable population. Confocal microscopic examination using several DNA and peptidoglycan stains did not provide enough resolution to determine if filaments contained multiple genome copies or lacked complete division septa (not shown). Future work investigating DNA replication and septation apparatus formation should yield additional insight into this aspect of RacRS function.

Using single cell analyses, we demonstrated that filamented ΔracR bacteria were more defective than their shorter counterparts for two key pathogenic attributes: motility and host cell invasion. Motility and chemotaxis defects are associated with decreased colonization in multiple animal models (28, 33, 46, 66, 73). Chemotaxis is important for the affinity of C. jejuni for the lower intestinal tract, where attractants such as carbon sources and electron donators and acceptors are abundant (31, 68). Motility may also be required to overcome the peristaltic action of the gastrointestinal tract, preventing the rapid
shedding of intestinal bacteria before successful colonization is established (28). In all organisms, body (or cell) size sets an energetic limit for movement (53), and alterations in size without a parallel differentiation of the movement apparatus will have a dramatic alteration on movement energetics. For instance, theoretical calculations have intimated that a 0.1 µm difference in bacterial diameter results in a 105-fold change in the energetic cost of chemotaxis (45). In ΔracR and ΔracS mutants, flagellar structure appeared unaffected, with all of the bacteria displaying two polar flagella which presumably generate the same motive force irrespective of bacterial size. Elongated bacteria were the slowest-swimming cells in broth culture, were unable to migrate from the zone of inoculation in semi-solid agar, and were also impaired in their ability to change direction. The elongated subset was also defective for invasion of tissue culture cells, an established in vitro marker for virulence. Several reasons for this can be envisaged. Increased swimming speed has been previously correlated with an elevated ability of C. jejuni to invade Caco-2 epithelial cells (65). While the exact mechanism remains to be elucidated, Szymanski et al. postulated that this may be the result of increases in impact during contact between the rapidly swimming bacteria and the host cell surface (65). It is also possible that invasion of elongated bacteria is physically impeded simply by virtue of their larger size, with increased eukaryotic cell membrane remodeling required to take up larger organisms potentially biasing towards invasion of primarily shorter cells.

Given our microscopy data and previously published findings (12), we were surprised that the 81-176 ΔracR mutant produced halo diameters approximately half the size as wt in semi-solid agar. It was important to establish whether this defect was strain-dependent, and also whether this reflected the swimming behaviour of the entire population or was an indirect effect such as decreased growth rate in viscous media or an effect of elongated bacteria “blocking” the outward migration of shorter cells. We first established that this defect was not specific to growth temperature or strain 81-176, as ΔracR mutants in strains 11168 and 81116 exhibited similar defects relative to the parent strain at both 38°C and 42°C, and comparison of halo diameters in various agar concentrations suggested motility rather than growth defects. Co-inoculation experiments also showed that elongated bacteria in the ΔracR mutant population did not inhibit the outward migration of motile wt (short) organisms in semi-solid motility agar.
Quantification established that *wt* individuals have a clear advantage in swimming speed over ∆racR. It is of note that in several bacterial species, altered levels of heat shock proteins can lead to a motility defect (5, 51, 54). Hypotheses to explain these findings have been proposed but await experimental validation. In support of the idea that chaperones influence motility, we observed that a ∆dnaJ mutant exhibited a modest decrease in motility at 38°C relative to the parent strain in semi-solid agar (data not shown). Of note, 3 of the 11 proteins previously identified as disregulated in ∆racR were sequenced and found to be RacR and two isoforms of a cytochrome *c* peroxidase (12). A different group showed that two periplasmic *C. jejuni* cytochrome *c* peroxidases are important for chicken colonization, but no *in vitro* roles have been identified (8). Future studies will investigate if disregulation of *dnaJ* or other genes in the RacR regulon have an impact on motility and other aspects important for colonization.

In our study, the ∆racR but not the ∆racS mutant had a modest growth defect at 42°C; however, similar chicken colonization defects in the absence of either *racR* or *racS* suggested that physiological defects other than the ability to grow at 42°C are associated with the two-component system mutants. Our findings highlight roles for the RacRS two-component system in overcoming stresses associated with the heat shock response (i.e., elevated temperature, osmolarity, and the ability to modulate HSP gene expression), influencing bacterial length, and maintaining motility. We hypothesize that a significant proportion of ∆racR and ∆racS mutant populations that are orally administered to chickens are unable to traverse the viscous mucus covering the intestinal epithelium, and are consequently both more subject to peristaltic expulsion and exposed for a longer period to stressors within the intestinal/cecal lumen which they are likewise compromised for surviving. In sum, our work shows the importance of RacRS on previously unidentified physiological aspects that likely collectively contribute to *C. jejuni* fitness *in vivo.*
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References


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*Abbreviations: chloramphenicol resistant (Cm<sup>K</sup>), kanamycin resistant (Kn<sup>R</sup>), streptomycin resistant (Str<sup>R</sup>).
Table 2. Bacterial length characteristics of *C. jejuni* wt and ∆racR and ∆racS mutants propagated at 38°C.

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<th>∆racS</th>
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<td>2228</td>
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<td>% &lt; 1.50 µm b</td>
<td>63.47</td>
<td>37.70</td>
<td>36.26</td>
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*The coefficient of variation (*Cv*) is a ratio of the standard deviation to the mean and provides a normalized measure of the population length distribution when comparing strains with different mean cell lengths.*

*bRefers to percent of the isogenic bacterial population that is less than 1.50 µm.*
Figure 1. In vitro growth curves of wt and mutant strains at 38°C and 42°C and colonization of day-of-hatch Leghorn chicks. The growth patterns of 81-176 wt, (●); ΔracR, (○); and ΔracS, (▼); are shown at (A) 38°C and (B) 42°C. Values shown are representative of three independent experiments. OD<sub>600</sub>, optical density 600 nm. (C) Chicks were orally inoculated with 10<sup>8</sup> CFU of <i>C. jejuni</i> resuspended in PBS. Six days post infection the birds were sacrificed. The bacterial load in the cecum was quantified and is represented as CFU g<sup>-1</sup> cecum. Each dot represents the bacterial load in one chicken, and the horizontal lines represent the mean.

Figure 2. Measurements of light production of promoter-luciferase fusions in <i>C. jejuni</i> and <i>E. coli</i>. The <i>racR</i> and <i>dnaj</i> transcriptional regulatory sequences were subcloned in front of the <i>luxCDABE</i> operon in pRY112 and the vector was transformed into <i>C. jejuni</i> wt, Δ<i>racR</i> and Δ<i>racS</i> mutants. Measurements were made for the first 12 hours of bacterial growth. Light counts per second (CPS) were normalized to the OD<sub>600</sub> at the time of measurement. The data for (A) <i>P<sub>racR</sub></i>-<i>luxCDABE</i> and (B) <i>P<sub>dnaj</sub></i>-<i>luxCDABE</i> in <i>C. jejuni</i> wt (●), Δ<i>racR</i> (○) and Δ<i>racS</i> (▼) are shown. Data are an average of 4 independent cultures, and standard deviation is denoted by error bars. (C) A map of the pRY112 derived vector utilized to investigate RacR dependent transcriptional regulation in <i>E. coli</i>. The luciferase operon was fused to <i>P<sub>dnaj</sub></i> in a plasmid with <i>racR</i> under transcriptional regulation of the arabinose-inducible <i>P<sub>bad</sub></i> promoter. Transcriptional expression of <i>araC</i> was under the constitutive promoter (<i>P<sub>c</sub></i>). (D) The light produced by <i>E. coli</i> cultures carrying the <i>P<sub>dnaj</sub></i>-<i>luxCDABE/araC</i> or <i>P<sub>dnaj</sub></i>-<i>luxCDABE/araC/P<sub>bad</sub> racR</i> vectors in the presence (+) or absence (-) of arabinose. Error bars denote standard deviation. ns, not significant.

Figure 3. Survival and colony formation of <i>C. jejuni</i> exposed to elevated growth temperatures or osmotic stress. The 81-176 wt, mutants (Δ<i>racR</i>, Δ<i>racS</i>), and complemented (Δ<i>racR</i><sup>C</sup>) strains were diluted to OD<sub>600</sub> of 0.1 and spot-plated on solid media with a serial dilution ranging from 10<sup>6</sup> to 10<sup>-5</sup> (denoted by the
vertical wedge). Images were captured after 48 hours of incubation at (A) the indicated temperatures on unsupplemented MH plates or (B) on MH plates supplemented with 0.8% NaCl at indicated temperatures, and are representative of three independent experiments.

**Figure 4.** Survival and colony formation of *C. jejuni* at elevated growth temperatures and the RT-qPCR analysis of *C. jejuni* exposed to a temperature increase from 38°C to 44°C. (A) The 81-176 wt, and mutants (ΔracR, ΔdnaJ, ΔdnaJ/ΔracR), were diluted to OD_{600} of 0.1 and spot-plated on solid media with a serial dilution ranging from 10^6 to 10^{-5} (denoted by the vertical wedge). Images were captured after incubation at indicated temperatures and are representative of three independent experiments. (B) *C. jejuni* 81-176 wt, and mutants (ΔracR, ΔracS) were grown to early log phase in MH broth at 38°C and shifted to 44°C for 15 minutes. The expression of *dnaJ*, *dnaK* and *groEL* was analyzed by RT-qPCR. Bars represent the expression ratio from strains shifted to 44°C relative to those that remained at 38°C, with *rpoA* used as an internal control. The horizontal line indicates no difference in expression between the experimental conditions. Data is representative of three independent experiments.

**Figure 5.** Analysis and quantification of *C. jejuni* cell length by light microscopy of bacterial populations. Representative images of strains (A) 81-176 wt and (B) ΔracR, and (C) distribution of the length of individual cells of wt, ΔracR, and ΔracS mutants cultured at 38°C. Over 2000 individual bacteria of each strain were examined from two individual experiments.

**Figure 6.** Investigating the impact of *C. jejuni* cell length in swimming speed and invasion of Caco-2 epithelial cells. (A) The swimming speed of 50 individual wt and ΔracR cells was examined by tracking swimming *C. jejuni* through MH broth by time-lapse microscopy. (B) wt and ΔracR strains expressing GFP were used to infect Caco-2 cells. The inset bacterium in the ΔracR mutant “media” panel is ~30 μm long. To differentiate between extracellular and intracellular bacteria in the cell-associated fractions, those
samples were exposed to an anti-\textit{C. jejuni} antibody either without permeabilization (only extracellular bacteria will be antibody-accessible) or following permeabilization with Triton X-100 (extracellular and intracellular bacteria will be antibody-accessible) and visualized using a secondary antibody conjugated to Alexa 568, which appears red. Depending on the relative ratio of GFP expression to antibody reactivity, antibody-accessible bacteria will appear red to yellow-orange. Caco-2 cell nuclei were stained with DAPI and are depicted by the color blue.

**Figure 7.** Motility analysis \textit{C. jejuni} \textit{wt}, \textit{ΔracR} and \textit{ΔracS} mutants. (A) Representative images of semi-solid motility plates incubated for 28 hours at 38°C of 81-176 \textit{wt}, \textit{ΔracR}, \textit{ΔracS} and \textit{ΔracR}C and (B) the halo diameter quantified. Values are an average of at least six independent experiments, and the standard deviation is represented by error bars. (C) Representative images of motility plates of \textit{wt} and \textit{ΔracR} mutants of \textit{C. jejuni} strains 11168 and 81116.

**Figure 8.** Population level analysis of \textit{C. jejuni} swimming speed through semi-solid media. (A) The \textit{ΔracR} mutant bacteria were sampled from either the outer (O), middle (M), or center (C), locations of the migration front of semi-solid motility agar. Elongated bacteria were localized at or near the zone of inoculation at the center of the motility halo and are indicated by white arrows. (B) The migration of \textit{wt} and \textit{ΔracR} through 0.5% and 0.6% agar. (C) \textit{wt} and \textit{ΔracR} were co-inoculated at a 1:1 ratio (\textit{wt}+\textit{ΔracR}) in a 0.4% agar motility plate and the halo diameter was compared to either strain inoculated alone. (D) Bacteria from the migration front of a \textit{wt} + \textit{ΔracR} co-inoculated culture were harvested and spot-plated on solid media with a serial dilution ranging from $10^0$ to $10^{-5}$ (denoted by the vertical wedge) on MH media or MH media supplemented with kanamycin. In all cases a representative image is shown.