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Differential Flagellin Expression in a *flaA flaB*$^+$ Mutant of *Campylobacter jejuni*

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***Campylobacter jejuni* 81116 has two genes coding for flagellin, *flaA* and *flaB*. Fully motile wild-type *C. jejuni* bacteria express the *flaA* gene, with no *flaB* message being detected. A nonmotile *flaA flaB*$^+$ mutant, R1, produced detectable levels of flagellin B which was incorporated into truncated flagella. After R1 had invaded INT-407 cells, a variant with increased motility, R1-V2, was isolated. R1-V2 produced full-length flagella and an increased amount of flagellin B. Transcriptional analysis showed that R1-V2 contained more *flaB* mRNA than its parental strain, R1. The *flaB* gene promoter sequence and primer extension experiments confirmed that transcription of the *flaB* gene is initiated from a σ^{54} promoter. Neither the promoter sequence nor the coding sequence of *flaB* had changed in R1-V2. In contrast to R1, R1-V2 no longer produced (truncated) *flaA* mRNA. The σ^{28} *flaA* promoter sequence was not changed in R1-V2. We propose that expression of the two flagellin genes in *C. jejuni* 81116 is regulated at the transcriptional level, in such a way that predominantly one gene at a time is transcribed. We compared the levels of invasiveness of the wild-type strain, R1, and R1-V2 for INT-407 cells. The shift in expression from *flaA* to *flaB* occurred not only during invasion assays but also under different conditions in the absence of eukaryotic cells.**

Campylobacter jejuni is a gram-negative bacterium that is an important enteropathogen of man and a number of animal species. The mechanisms by which *C. jejuni* causes disease involve colonization, attachment, invasion, and toxin production. Functional flagella are important as virulence factors. Aflagellate bacteria do not colonize in vivo (1, 19, 21), and flagellar mutants are less invasive in vitro (9, 28). In addition, human volunteers dosed with a mixture of motile and nonmotile variants of *C. jejuni* excrete only motile bacteria (5).

The flagellin gene organizations in two strains of *C. jejuni* and one *Campylobacter coli* strain have been determined, and in all three cases two flagellin genes are present on the genome (8, 11, 24). The complete nucleotide sequences of the two genes of *C. jejuni* 81116 and *C. coli* VC167 have been determined (11, 24). In both species the two genes are arranged head-to-tail in the same direction, separated by 174 bp. The coding regions of *flaA* and *flaB* appear to be almost identical. However, each gene contains its own, different promoter; a recognition site for the alternative sigma factor σ^{28} is present upstream of *flaA*, and a putative promoter consensus sequence for sigma-54 has been identified upstream of *flaB* (11, 14, 24). These features suggest that the two flagellin genes are subject to differential regulation. Although antigenic variation of *Campylobacter* flagella has been reported (2, 12), the functional significance of flagellin gene duplication is not yet understood.

Expression of the two flagellin genes in both *Campylobacter* spp. has been studied by transcriptional and mutational analysis. In *C. coli*, transcripts of both genes are detected, but *flaA* is expressed at a higher level than *flaB* (11). Inactivation of *flaB* leads to slightly less motile mutants of this species, while inactivation of *flaA* results in mutants with reduced motility and truncated flagella (10). Electron microscopic studies using flagellin A-adsorbed antiserum show that flagellin B is incorporated in the flagellum of wild-type (WT) *C. coli* (10). In flagellated WT *C. jejuni* bacteria, no transcripts of *flaB* are detected (24) and *flaB* can be inactivated without a loss of motility (28). *flaA* mutants of *C. jejuni* 81116 are relatively nonmotile and produced truncated flagella composed of flagellin B (28).

To date, it has remained unclear whether, and under what conditions, the *flaB* gene of *Campylobacter* spp. can produce functional full-length flagella composed only of flagellin B. This study presents evidence that a *flaA flaB*$^+$ mutant of *C. jejuni* is able to synthesize such flagellin B flagella. The mutant was isolated after invasion of INT-407 cells. We studied *flaA* and *flaB* expression in this variant at the transcriptional level, and we examined the effect of the flagellin B phenotype on invasiveness in vitro.

MATERIALS AND METHODS

Bacteria and culture conditions. The origin of *C. jejuni* 81116 and the construction of mutants R1 and R3 have been described previously (28). Unless otherwise specified, bacteria were cultured on saponin plates at 42°C as described by Nuijten et al. (23). Colony size was determined by culturing in semisolid thioglycolate plates containing 0.4% agar (6). R1-V2 was isolated from R1 bacteria which had invaded INT-407 cells.

Invasion of INT-407 cells. The INT-407 cell line, derived from human embryonal tissue of the jejunum and ileum, was obtained from Flow Laboratories. The technique of *Campylobacter* invasion has been described in detail elsewhere (28). Briefly, monolayers (10^6 cells) were washed with Dulbecco's

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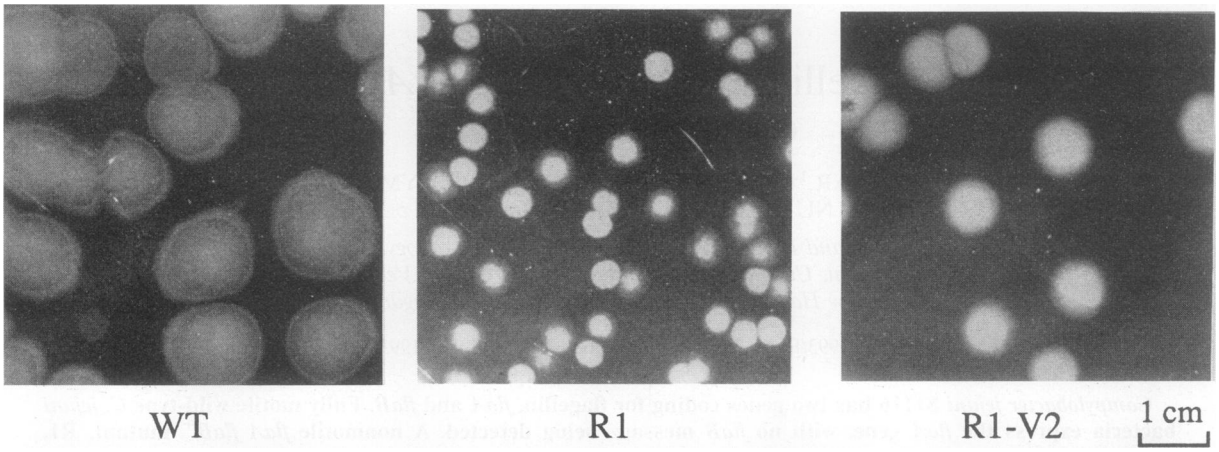

FIG. 1. Colony size and morphology of WT *C. jejuni* 81116, mutant R1, and variant R1-V2 in semisolid thioglycolate-agar plates.

modified Eagle medium (DMEM) 30 min prior to inoculation with bacteria (multiplication of infection of 10^3 bacteria per cell). Infected monolayers were incubated at 37°C in 5% CO_2–95% air. After several time intervals (see Fig. 6), monolayers were washed five times with DMEM and reincubated for another 3 h with DMEM containing 250 μg of gentamicin per ml. Then, cells were washed three times with phosphate-buffered saline and lysed in 0.5% Triton X-100. Suspensions were plated on saponin agar medium, and the numbers of CFU were counted and calculated. After invasion of INT-407 cells by R1, the increased motility of R1-V2 (swarming colonies) was only observed on freshly made saponin plates.

Western blots (immunoblots). Preparation of bacterial protein extracts, gel electrophoresis, blotting procedures, and immunostaining were performed by the method of Wassenaar et al. (28). The production and characterization of monoclonal antibodies CF1 and CF17 have been described previously (20, 25).

Northern (RNA) blots. RNA was isolated from overnight cultures of *C. jejuni* by using RNAzol (Cinna Biotecx Laboratories Inc.). Total RNA concentrations were measured by the A_{260}. RNA gel electrophoresis, Northern blotting, and hybridization were performed as described before (23). Hybond-N filters were used, and the RNA was cross-linked to the filters by UV irradiation. A mixture of the following three fragments was used as a full-length flagellin-specific probe recognizing *flaA* and *flaB* together: the 650-bp *Ssp*I-*Bgl*II fragment of pIVB3-304, containing the first part of *flaA*, and the 1.2- and 1.27-kb *Bgl*II-*Pst*I fragments of clone pIVB3-300, containing the largest parts of both genes (24). The fragments were radioactively labelled by using random primers. Probes specific for *flaA* or *flaB* were prepared by radioactive PCR (see below).

PCR and sequencing reactions. PCRs were performed on *C. jejuni* DNA isolated according to the method of King et al. (16). The following primers were used: primer A1 (5′-CGTAT TAACACAAATGTTGCAGCATTG-3′), which is identical to nucleotides +10 to +36 of *flaA* (the initiating ATG codon starts at +1); primer A2 (5′-AGCATCTAAACTTTTAG CATTAAG-3′), which is complementary to positions +60 to +83 of *flaA*; primer B1 (5′-TTAATGTTAAAGCAGCAG-3′), which is located within the coding region of *flaA* at nucleotide positions −329 to −312 of *flaB*; primer B2 (5′-AGGATAAAC ACCAACATCGGTGCATTA-3′), which is identical to nucleotides +10 to +36 of *flaB*; primer B3 (5′-CTTGTCAAGTTC ATTAGAGTTAAC-3′), which is complementary to positions +60 to +83 of *flaB*; and primer C (5′-ACCAACACCTA TATCTCC-3′), which is complementary to both flagellin genes at positions +931 to +948. All primers were purchased from Pharmacia. PCRs were performed by using *Taq* DNA polymerase purchased from Promega with 35 cycles of 1 min at 90°C, 2 min at 55°C, and 3 min at 72°C.

PCR fragments were cloned into pBluescript II KS(+) (Stratagene) which had been digested with *Eco*RV and dephosphorylated by using calf intestine alkaline phosphatase (Boehringer). Three clones of each fragment were sequenced by using the AutoRead Sequencing Kit and the Automated Laser Fluorescent DNA Sequencer (Pharmacia LKB).

To prepare a radioactive probe specific for *flaA*, primers A1 and A2 were used, and for a *flaB*-specific probe, primers B2 and B3 were used. The PCRs were performed in the presence of 0.2 mM deoxynucleoside triphosphates (dNTP) supplemented with 10 mCi of [α-^{32}P]dATP (3,000 Ci/mmol).

Primer extension. To map the 5′ ends of the flagellin messengers, primers A2 (complementary to the *flaA* mRNA) and B3 (complementary to the *flaB* mRNA) were used. The primers were radioactively labelled with [γ-^{32}P]ATP by using T4 polynucleotide kinase. Thirty nanograms of labelled primer (600 cpm/ng) was annealed to 10 μg of denatured bacterial RNA at 42°C for 1 h in 100 mM Tris-HCl (pH 8.3)–140 mM KCl. Reverse transcriptase (10 U of Superscript RT; Bethesda Research Laboratories) and 0.5 mM each dNTP were added, and the reaction was continued for 30 min at 42°C.

RESULTS

Isolation of the motility variant R1-V2 from mutant R1. *C. jejuni* 81116 mutant R1 (*flaA flaB*$^+$) is relatively nonmotile and is 100-fold less invasive for INT-407 cells than the motile WT strain (24). Ten to twenty percent of the colonies obtained from those R1 bacteria that had nevertheless invaded INT-407 cells appeared as swarming colonies on fresh saponin plates. Figure 1 shows the motility of these phenotypic variants in semisolid thioglycolate–0.4% agar plates. The motility of the variant was intermediate between that of the WT strain and that of R1 (Fig. 1), and the variant was designated R1-V2. Four distinct R1-V2 colonies were selected for further investigation. After these colonies were subcultured or stored at −80°C, 30 to 60% of the offspring colonies from these selected bacteria reverted back to the R1 motility phenotype. Repeated selection of R1-V2 colonies from thioglycolate plates resulted

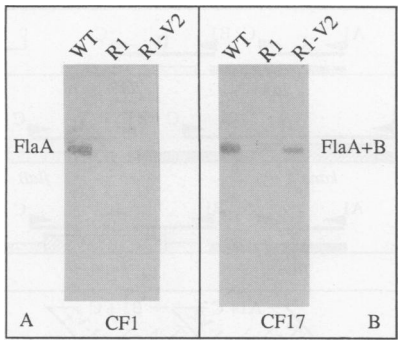

FIG. 2. Western blots of total bacterial protein extracts reacted with monoclonal antibodies CF1, specific for flagellin A (A), and CF17, recognizing both flagellin A and flagellin B (B). Panels A and B contain total protein profiles from the WT strain, R1, and R1-V2.

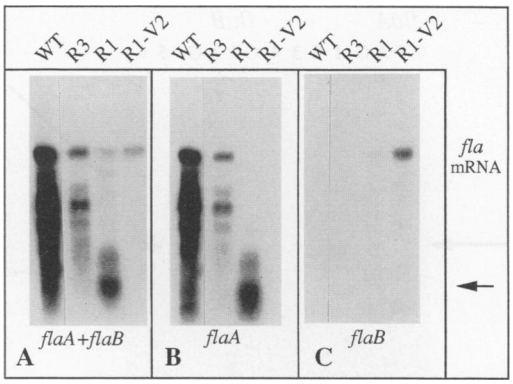

FIG. 3. Northern blots of total bacterial RNA isolated from the WT strain, R3, R1, and R1-V2. Each lane contains 10 μg of total RNA. The position of the full-length flagellin mRNA is shown (*fla* mRNA), and the position of the truncated *flaA* mRNA of R1 is indicated by the arrow. Blots in panel A were hybridized with a full-length probe specific for flagellin genes A and B, and blots in panels B and C were hybridized with 5′-proximal probes specific for flagellin A and flagellin B, respectively.

in a clonal population that was stable upon culture, and this population was used for further experiments. R1-V2 bacteria possess long flagella, which appeared to be more fragile than those of WT bacteria but were otherwise undistinguishable when observed by electron microscopy (data not shown).

Since bacteria with the R1-V2 phenotype had to be subcultured several times before they remained stable, there was a possibility that we selected for a mutant having the R1-V2 phenotype. Therefore, R1 and the stable form of R1-V2 were maintained in Luria-Bertani broth for 72 h at 42°C without shaking and then plated in thioglycolate plates. Incubation in Luria-Bertani broth is completely different from incubation under the conditions that we use to grow *C. jejuni* 81116. Although the frequency of switching was highly variable from experiment to experiment, the R1-V2 bacteria previously considered stable changed into R1, and vice versa. Cultivation of R1 under standard conditions had no effect on the phenotype. Thus, we believe that stable R1-V2 is not a mutant but rather is a variant. In addition, a variant with an R1-V2 phenotype (WT-V2) was isolated from WT bacteria by using this Luria-Bertani broth-induced shift. However, this variant was still unstable after several in vitro passages and was not used for further studies.

Identification of the R1-V2 flagellin. The nature of the flagellin produced by R1-V2 was determined by using monoclonal antibodies. A Western blot containing total bacterial protein extracts of the WT strain, R1, and R1-V2 was reacted with monoclonal antibody CF1, which is specific for flagellin A of *C. jejuni* 81116 (25). Flagellin A was detected only in the lane containing protein extract of WT bacteria (Fig. 2A). Monoclonal antibody CF17 recognizes a different epitope, which is present on both flagellins, A and B, and this antibody detected flagellin in the WT strain and R1-V2, but it detected only minor amounts of flagellin in R1 (Fig. 2B). These results show that R1-V2 contained flagellin B only and contained larger amounts of it than R1 did.

In order to determine whether the changed phenotype of R1-V2 always coincided with increased expression of *flaB*, six independent stable R1-V2 isolates were obtained from R1 bacteria that had invaded INT-407 cells. Total protein extracts were analyzed by Western blotting, and in all six isolates flagellin B exclusively was detected, at a level comparable to that for R1-V2 and higher than that for R1 (results not shown). For all further experiments, one of these isolates was used.

To investigate whether other proteins are coregulated with flagellin B in R1-V2, total soluble protein extracts of WT, R1, and R1-V2 bacteria were analyzed by two-dimensional electrophoresis. The results (data not shown) did not reveal detectable differences in protein profiles.

Differential expression of *flaA* and *flaB* in R1 and R1-V2. The mRNA levels of *flaA* and *flaB* were determined by Northern blotting of total bacterial RNA using three probes. The first probe was specific for both *flaA* and *flaB* mRNA (Fig. 3A), the second probe was specific for the 5′ end of *flaA* mRNA (Fig. 3B), and the third probe was specific for the 5′ end of *flaB* mRNA (Fig. 3C). Mutant R3 contains a kanamycin resistance gene inserted in the *flaB* gene (*flaA*$^+$ *flaB*) (28) and was included as a control. The probe detecting both messages hybridized to RNA isolated from WT, R3, and R1-V2 bacteria and in lower amounts to RNA isolated from R1 bacteria (Fig. 3A). When the same RNA was hybridized with a 5′-proximal probe specific for *flaA* mRNA (Fig. 3B), no hybridization was observed in the R1-V2 lane and no full-length *fla* message was seen in the R1 lane. A truncated *flaA* mRNA was detected in R1 (Fig. 3B). This truncation of *flaA* mRNA is caused by the insertion of the kanamycin resistance gene into the *flaA* gene of R1. Both the probe recognizing *flaA* and *flaB* mRNA and the *flaA*-specific probe showed degradation of the *flaA* message. A 5′-proximal probe specific for *flaB* (Fig. 3C) detected mRNA in R1-V2, and in minor amounts in R1, but not in the WT strain or R3. These results indicate that R1-V2 differs from R1 by an increased level of *flaB* mRNA concomitant with a decrease in the (truncated) *flaA* mRNA level.

Primer extension experiments confirmed that the *flaB* gene in R1-V2 is transcribed from a sigma-54-type promoter (Fig. 4). The *flaB*-specific primer produced an elongated product with RNA isolated from R1-V2; little product was detectable in R1. Promoters recognized by sigma-54 have a highly conserved sequence motif with a consensus TGGCAC-N_5-TTGC around positions −26 to −12 (18). This motif, differing in only one nucleotide, is present upstream of *flaB* (Fig. 4).

Organization of the flagellin genes and the nucleotide sequences of their promoter regions in R1-V2. Southern analysis of chromosomal DNA digests of R1-V2 and R1, hybridized with probes specific for flagellin or kanamycin sequences, resulted in identical patterns, indicating that the kanamycin resistance gene in R1-V2 was still inserted in *flaA* (data not shown). To determine whether the increased level of

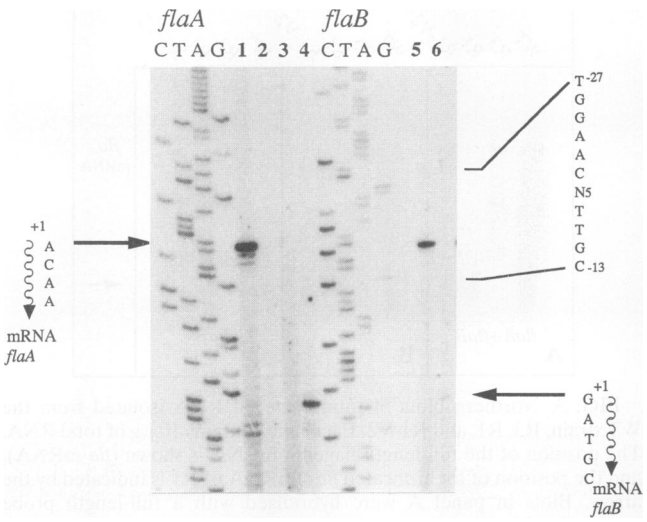

FIG. 4. Determination of the 5′ ends of flagellin mRNAs by primer extension. RNA isolated from the WT strain and a labelled primer specific for *flaA* mRNA were included as controls. RNAs isolated from the WT strain (lanes 1 and 3), R1-V2 (lanes 2 and 4), and R1 (lanes 5 and 6) were hybridized with primer A2, specific for *flaA* mRNA (lanes 1, 2, and 5), or with primer B3, specific for *flaB* mRNA (lanes 3, 4, and 6). Straight arrows indicate the positions of the transcription start sites of *flaA* (at the left) and *flaB* (at the right). The consensus sigma-54 recognition sequence is indicated. DNAs derived from the WT strain (on the left) and R1-V2 (on the right) were first amplified by PCR and then sequenced with primers A2 and B3, respectively, and are included on the gel for comparison.

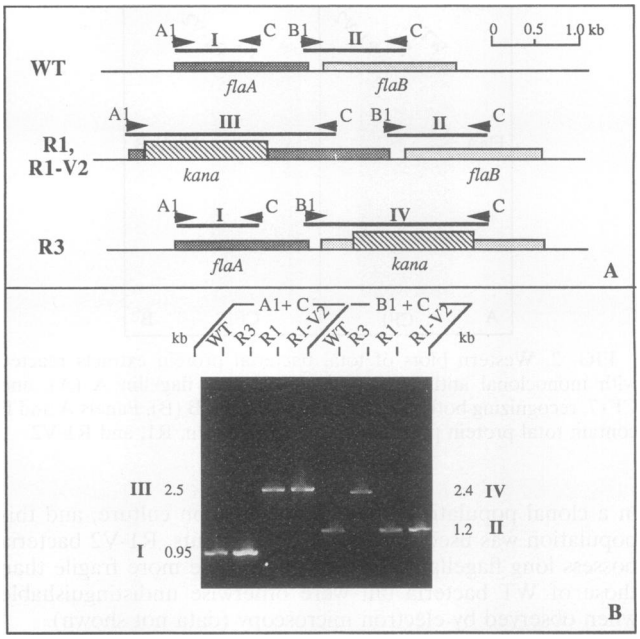

FIG. 5. Flagellin gene organization in R1-V2. (A) Schematic representation of the flagellin genes of the WT strain, R1, R1-V2, and R3. Primers A1, B1, and C are indicated by arrowheads according to their orientations. PCRs using these primers result in fragments I to IV, which are represented as solid lines. (B) Ethidium bromide-stained agarose gel containing PCR products obtained with WT, R3, R1, and R1-V2 chromosomal DNAs by using oligonucleotides A1 and C (left side) and B1 and C (right side). Fragments I to IV correspond to the fragments in panel A. Lengths are given in kilobase pairs.

flaB transcripts was the result of a recombinational event within the flagellin genes of R1-V2, this region of the genome was amplified by PCR and sequenced. Two PCRs were carried out on DNA isolated from R1-V2, the WT strain, R1, and R3. The results (Fig. 5) show that a PCR with primers specific for *flaA* amplifies a 2.5-kb fragment in R1 and R1-V2, in contrast to the 0.95-kb fragment obtained from the WT strain and mutant R3. This 2.5-kb fragment is of the expected size in view of the kanamycin resistance gene insertion in the *flaA* genes of R1 and R1-V2. The PCR products obtained with primers specific for *flaB* are identical in length for the WT strain, R1, and R1-V2. The *flaB* PCR product of R3 is 2.4 kb long, as expected on the basis of the strategy that was used to construct the R3 mutant (i.e., the partial deletion of an internal part of the *flaB* gene and the insertion of the kanamycin resistance gene).

The PCR products obtained with primers B1 and C were used to determine the nucleotide sequence of the promoter and the 5′ end of *flaB*; the sequences of R1 and R1-V2 are identical to the WT sequence, including its putative sigma-54-dependent promoter (24) (see Fig. 4). Thus, the change in the *flaB* mRNA level of R1-V2 is not caused by DNA rearrangements or mutations in the *flaB* promoter region.

In order to exclude possible mutations within the *flaB* gene that could be the cause of a more stable mRNA, a more stable FlaB protein, or a more stable flagellar filament, the entire *flaB* coding region of R1-V2 was amplified by PCR and sequenced. The *flaB* sequences of R1, R1-V2, and the WT strain were identical (data not shown).

Since *flaA* transcription is decreased in R1-V2, the nucleotide sequence of the *flaA* promoter of R1-V2 was determined. A DNA fragment was amplified from positions −330 to +948 (the ATG codon being at position +1). This PCR product was sequenced from positions +1 to +85 by using the *flaA*-specific A1 primer. In this region, including the σ^{28} promoter consensus sequences (24), no changes were observed.

Invasiveness of R1-V2 in vitro. The invasive properties of *Campylobacter* spp. can be studied in vitro by using monolayers of INT-407 cells. In this assay, invasion is the combined process of bacterial attachment to and penetration into cells. Previous studies demonstrated that the mutant R1 is less invasive than the WT strain and that invasion is partly dependent on motility (9, 28). It seemed likely, therefore, that the poor invasiveness of R1 was a reflection of the truncated flagella. Since R1-V2 was isolated from R1 after the invasion of INT-407 cells, it was of interest to determine whether R1-V2 was more capable of invading these cells. Therefore, the levels of invasiveness of the WT strain, R1, and R1-V2 were compared. As shown in previous experiments, the number of WT organisms reached a maximum of about 5×10^5 invading bacteria per 10^6 cells after 3 h. In contrast, R1 and R1-V2 invaded to maximum levels of only 1.2×10^3 and 2.5×10^3 bacteria per 10^6 INT-407 cells, respectively (Fig. 6). These results indicate that the increased motility conferred by the full-length flagella of R1-V2 had only a minor positive effect on invasiveness. Nevertheless, the invasiveness of both of these mutants can be enhanced 10-fold (Fig. 6) by centrifugation of the bacteria onto the monolayer, presumably because of the improved contact between the bacteria and the cells. However, the levels of invasiveness still remained 10-fold lower than that of the WT strain. Centrifugation had no effect on the invasiveness of the WT strain.

In a control invasion assay with R1 the INT-407 cells were omitted; in this case no R1-V2 was detected. In addition, R1-V2 was never found to be present in R1 cultures grown

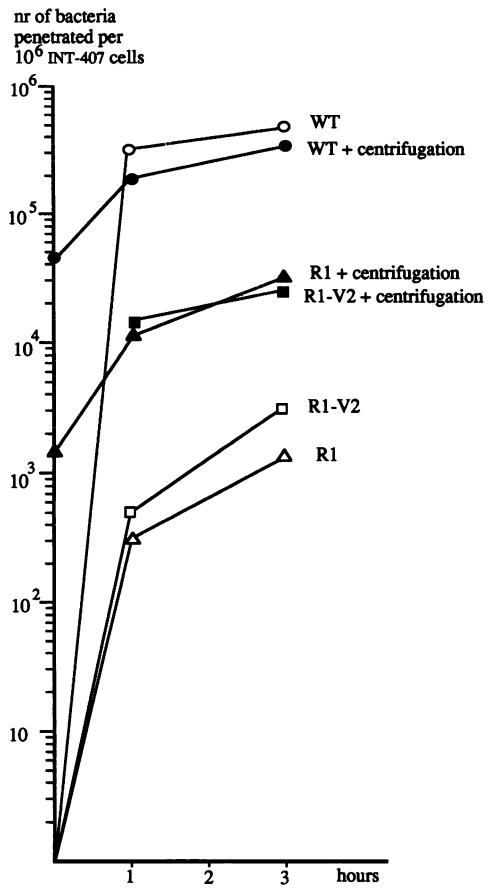

FIG. 6. Invasion of INT-407 cells by the WT strain, R1, and R1-V2. Bacteria were added to the cells (open symbols) or were added to the cells and also centrifuged onto the cells (closed symbols). For the latter case the sample taken directly after centrifugation is plotted at 0 h. nr, number.

under standard growth conditions (at least 10^7 bacteria examined) prior to inoculation of INT-407 cells.

DISCUSSION

Flagella are known to have a significant role in the pathogenicity of the thermophilic *Campylobacter* spp. The discovery of flagellin gene duplication in *C. jejuni* 81116 suggests that either recombinational events between the two genes (4) or differential expression of the two genes, each one having its own, different promoter, may lead to production of different types of flagella. These mechanisms may provide the bacterium with the ability to respond to an environment changing, for example, with regard to pH, concentrations of salts, viscosity of the medium, or neutralizing antibodies in a host. Prior to this study, only expression of flagellin A and the concomitant synthesis of FlaA flagella in *C. jejuni* 81116 had been shown to be essential for invasion of INT-407 cells (28). *flaB* expression had been observed only at low levels in a *flaA flaB*$^+$ mutant that produced truncated flagella (9, 28). In this study a variant expressing full-length flagellin B flagella (R1-V2) has been isolated from tissue culture cells after invasion by the *flaA flaB*$^+$ mutant R1. R1-V2 provided a suitable mutant for study of the expression of flagellin A and flagellin B and for study of the roles of the two flagellins and the two flagellum types in motility and invasion.

R1-V2 has motility intermediate between that of the WT strain and that of R1. Apparently, flagellin B flagella confer less motility on the bacteria than flagella composed of flagellin A. This suggests that the decreased motility is an intrinsic property of flagellin B. Analysis of *Salmonella* flagellin mutants having a changed motility or flagellar morphology indicated that mutations causing these effects are located within the C- and N-terminal regions of the flagellin (15). The differences between the FlaA and FlaB amino acid sequences are primarily within these terminal regions. Differential expression of *flaA* and *flaB* may give rise to the formation of either FlaA or FlaB flagella, each one having its own optimal function under specific, yet different, environmental conditions. R1-V2 may be more motile than was observed in this study under other circumstances, provided that FlaB is still expressed at a level high enough to allow formation of full-length flagella. Different degrees of motility within one *C. jejuni* strain, dependent upon the viscosity of the medium (7), have been described.

Differential flagellin gene expression in *Campylobacter* spp. has not been described previously. Alm et al. (3) reported that the activity of the *flaB* promoter in *C. coli* VC167 is affected by environmental conditions, but they demonstrated no effect on *flaA* transcription or the presence of full-length FlaB flagella. In this study we showed that *flaB* mRNA is not detectable in the WT strain but is present in R1-V2 and that *flaA* (truncated) mRNA is absent in R1-V2 while it is abundantly present in the WT strain. A significant change in messenger stabilities could account for these differences in RNA levels, but a more likely explanation is a change in transcriptional regulation. Flagellin gene expression seems to be regulated in such a way that one gene is transcribed at a time. Only the R1 (*flaA flaB*$^+$) mutant produces some *flaB* mRNA, which may be due to the absence of FlaA protein. The standard laboratory culture conditions for growing and maintaining *C. jejuni* 81116 appear to induce *flaA* expression and repress *flaB* expression. A variant with the R1-V2 phenotype, expressing the *flaB* gene, was induced during the invasion assay. R1-V2 was not present in R1 cultures prior to invasion. R1-V2 was not observed when an invasion experiment without INT-407 cells was performed. This strongly suggests that the microenvironment of the eukaryotic cell in tissue culture causes a change in expression leading to *flaB* transcription and a shutdown of *flaA* transcription. The finding that this shift also occurred under other conditions in the absence of eukaryotic cells indicates that the bacterium is responding to a stimulus or stimuli in the microenvironment and not to the eukaryotic cell itself. Therefore, the stability or instability of the R1-V2 variant also seems to be a direct result of the culture conditions.

We have shown here that, in contrast to the flagellar switch described for *Salmonella* spp. (26), the altered *flaA* and *flaB* expression in *C. jejuni* 81116 (R1 and R1-V2) does not result from DNA rearrangement in the flagellin genes or their promoters. Our data indicate that the *flaB* messenger in *C. jejuni* 81116 is produced from a sigma-54-dependent promoter similar to that found in *C. coli* (11). Besides *Campylobacter* spp., several other bacteria contain flagellin genes that are regulated by sigma-54-dependent promoters (13), e.g., *Caulobacter crescentus* (22) and *Pseudomonas aeruginosa* (27). Such promoters are usually dependent on, in addition to the sigma-54 protein, a *trans*-acting factor (such as NIFA or NTRC) which in turn can be facultatively expressed or activated under certain conditions (17). Changes in expression or properties of such *trans*-acting factors in R1 may have led to the appearance of R1-V2 by inducing *flaB* transcription. Simultaneously, *flaA*

expression was reduced, a process which might be directly or indirectly linked to regulation of *flaB* expression. Since the R1-V2 phenotype is not caused by a simple (point) mutation, we assume the existence of a complex molecular mechanism causing the events observed in flagellin gene expression. Although two-dimensional gel electrophoresis of total protein extracts of R1, WT, and R1-V2 bacteria did not reveal significant differences among these strains, regulatory proteins that are present in minor amounts could be undetectable by such procedures.

C. jejuni isolates or mutants expressing either FlaA or FlaB flagella do not differ only in motility but also in invasiveness in vitro: R1-V2 is 50-fold less invasive than the WT strain, regardless of the full-length flagella of R1-V2. Invasiveness is the combination of adherence and penetration, and it is dependent on motility. The contact between bacteria and cells can be enhanced in vitro by centrifugation, but this improves the invasiveness of R1 and R1-V2 to only a limited extent. Thus, if flagella play a role in adherence or penetration, then flagellin A flagella are more effective in these processes than flagellin B flagella. Colonization experiments using chickens, a natural host of *C. jejuni*, also showed that *C. jejuni* mutants having inactivated *flaA* genes were 100 to 1,000 times less efficient than WT strains, regardless of the motility conferred by truncated or full-length FlaB flagella (29). On the basis of these data and with the help of these flagellin mutants, investigations into the role of differential flagellin gene expression in infection of a susceptible host can now begin.

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REFERENCES

1. **Aguero-Rosenfeld, M. E., X.-H. Yang, and I. Nachamkin.** 1990. Infection of Syrian hamsters with flagellar variants of *Campylobacter jejuni*. Infect. Immun. **58:**2214–2219.
2. **Alm, R. A., P. Guerry, M. E. Power, and T. J. Trust.** 1992. Variation in antigenicity and molecular weight of *Campylobacter coli* VC167 flagellin in different genetic backgrounds. J. Bacteriol. **174:**4230–4238.
3. **Alm, R. A., P. Guerry, and T. J. Trust.** 1993. The *Campylobacter* σ^{54} *flaB* flagellin promoter is subject to environmental regulation. J. Bacteriol. **175:**4448–4455.
4. **Alm, R. A., P. Guerry, and T. J. Trust.** 1993. Significance of duplicated flagellin genes in Campylobacter. J. Mol. Biol. **230:**359–363.
5. **Black, R. E., M. M. Levine, M. L. Clements, T. P. Hughes, and M. J. Blaser.** 1988. Experimental *Campylobacter jejuni* infections in humans. J. Infect. Dis. **157:**472–479.
6. **Caldwell, M. B., P. Guerry, E. C. Lee, J. P. Burans, and R. I. Walker.** 1985. Reversible expression of flagella in *Campylobacter jejuni*. Infect. Immun. **50:**941–943.
7. **Ferrero, R. L., and A. Lee.** 1988. Motility of *Campylobacter jejuni* in a viscous environment: comparison with conventional rod-shaped bacteria. J. Gen. Microbiol. **134:**53–59.
8. **Fischer, S. H., and I. Nachamkin.** 1991. Common and variable domains of the flagellin gene, *flaA*, in *Campylobacter jejuni*. Mol. Microbiol. **5:**1151–1158.
9. **Grant, C. C. R., M. E. Konkel, W. Cieplack, Jr., and L. S. Tompkins.** 1993. Role of flagella in adherence, internalization, and translocation of *Campylobacter jejuni* in nonpolarized and polarized epithelial cell cultures. Infect. Immun. **61:**1764–1771.
10. **Guerry, P., R. A. Alm, M. E. Power, S. M. Logan, and T. J. Trust.** 1991. Role of two flagellin genes in *Campylobacter* motility. J. Bacteriol. **173:**4757–4764.
11. **Guerry, P., S. M. Logan, S. Thornton, and T. J. Trust.** 1990. Genomic organization and expression of *Campylobacter* flagellin genes. J. Bacteriol. **172:**1853–1860.
12. **Harris, L. A., S. M. Logan, P. Guerry, and T. J. Trust.** 1987. Antigenic variation of *Campylobacter* flagella. J. Bacteriol. **169:**5066–5071.
13. **Helmann, J. D.** 1991. Alternative sigma factors and the regulation of flagellar gene expression. Mol. Microbiol. **5:**2875–2882.
14. **Helmann, J. D., and M. J. Chamberlin.** 1988. Structure and function of bacterial sigma factors. Annu. Rev. Biochem. **57:**839–872.
15. **Kanto, S., H. Okino, and S.-I. Aizawa.** 1991. Amino acids responsible for flagellar shape are distributed in terminal regions of flagellin. J. Mol. Biol. **219:**471–480.
16. **King, V., T. Wassenaar, B. A. M. Van der Zeijst, and D. G. Newell.** 1991. Variations in *Campylobacter jejuni* flagellin, and flagellin genes, during in vivo and in vitro passage. Microb. Ecol. Health Dis. **4:**135–140.
17. **Kustu, S., E. Santero, J. Keener, D. Popham, and D. Weiss.** 1989. Expression of sigma-54 (*ntrA*)-dependent genes is probably united by a common mechanism. Microbiol. Rev. **53:**367–376.
18. **Morett, E., and M. Buck.** 1989. In vivo studies on the interaction of RNA polymerase sigma-54 with the *Klebsiella pneumoniae* and *Rhizobium meliloti* NifH promoters. The role of NifA in the formation of an open promoter complex. J. Mol. Biol. **210:**65–77.
19. **Morooka, T., A. Umeda, and K. Amako.** 1985. Motility as an intestinal colonization factor for *Campylobacter jejuni*. J. Gen. Microbiol. **131:**1973–1980.
20. **Newell, D. G.** 1986. Monoclonal antibodies directed against the flagella of *Campylobacter jejuni*: production, characterization and the lack of effect on the colonization of infant mice. J. Hyg. Camb. **96:**131–141.
21. **Newell, D. G., H. McBride, and J. M. Dolby.** 1985. Investigations on the role of flagella in the colonization of infant mice with *Campylobacter jejuni* and attachment of *Campylobacter jejuni* to human epithelial cell lines. J. Hyg. Camb. **95:**217–227.
22. **Ninfa, A. J., D. A. Mullin, G. Ramakrishnan, and A. Newton.** 1989. *Escherichia coli* σ^{54} RNA polymerase recognizes *Caulobacter crescentus flbG* and *flaN* flagellar gene promoters in vitro. J. Bacteriol. **171:**383–391.
23. **Nuijten, P. J. M., N. M. C. Bleumink-Pluym, W. Gaastra, and B. A. M. van der Zeijst.** 1989. Flagellin expression in *Campylobacter jejuni* is regulated at the transcriptional level. Infect. Immun. **57:**1084–1088.
24. **Nuijten, P. J. M., F. J. A. M. van Asten, W. Gaastra, and B. A. M. van der Zeijst.** 1990. Structural and functional analysis of two *Campylobacter jejuni* flagellin genes. J. Biol. Chem. **265:**17798–17804.
25. **Nuijten, P. J. M., B. A. M. van der Zeijst, and D. G. Newell.** 1991. Localization of immunogenic regions on the flagellin proteins of *Campylobacter jejuni* 81116. Infect. Immun. **59:**1100–1105.
26. **Silverman, M., and M. Simon.** 1980. Phase variation: genetic analysis of switching mutants. Cell **19:**845–854.
27. **Totten, P. A., J. C. Lara, and S. Lory.** 1990. The *rpoN* gene product of *Pseudomonas aeruginosa* is required for expression of diverse genes, including the flagellin gene. J. Bacteriol. **172:**389–396.
28. **Wassenaar, T. M., N. C. M. Bleumink-Pluym, and B. A. M. van der Zeijst.** 1991. Inactivation of *Campylobacter jejuni* flagellin genes by homologous recombination demonstrates that *flaA* but not *flaB* is required for invasion. EMBO J. **10:**2055–2061.
29. **Wassenaar, T. M., B. A. M. van der Zeijst, R. Ayling, and D. G. Newell.** 1993. Colonization of chicks by motility mutants of *Campylobacter jejuni* demonstrates the importance of flagellin A expression. J. Gen. Microbiol. **139:**1171–1175.