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The *Campylobacter* σ^{54} *flaB* Flagellin Promoter Is Subject to Environmental Regulation

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The complex flagellum of *Campylobacter coli* VC167 is encoded by two tandemly oriented flagellin genes which are transcribed as two discrete transcriptional units from two different classes of promoters. The *flaB* gene, which encodes the minor FlaB filament protein, is controlled by a σ^{54} promoter. A transcriptional fusion between a promoterless chloramphenicol acetyltransferase (CAT) reporter gene cartridge and *C. coli* VC167 DNA carrying *flaB* transcription and translation signals, including the typical position −13-to-−26 *flaB* σ^{54} consensus promoter sequence, was constructed. When carried on plasmid pRIC1013, the σ^{54}-CAT fusion expressed chloramphenicol resistance in *Escherichia coli*, and CAT production was affected by the pH of the growth medium, the composition of the growth atmosphere, and the growth temperature, with production being significantly higher at 42°C. A conjugative suicide vector, pRIC1028, containing the σ^{54}-CAT fusion was constructed and used to recombine the *flaB*-CAT fusion back into the *C. coli* chromosome in the correct position with respect to the *flaA* gene and its transcription terminator. CAT production from the *flaB* σ^{54} promoter in the *C. coli* transconjugant VC167-T2/28-1 was shown to peak at mid-log phase and to be modulated by growth medium pH, growth temperature, and the concentration of certain inorganic salts and divalent cations in the growth medium. Under growth conditions which promoted elevated *flaB* σ^{54} promoter activity, a *flaA flaB*$^+$ mutant of *C. coli* VC167 produced increased amounts of FlaB flagellar protein and displayed increased motility.

The thermophilic gram-negative spiral bacteria *Campylobacter coli* and *Campylobacter jejuni* are common causative agents of severe diarrhea in humans (6, 40, 43). These small spiral bacteria have a single polar flagellum at one or both ends of the cell, and the spiral shape of the bacteria and the motility imparted by the flagellum appear to play important roles in the ability of *Campylobacter* spp. to colonize the viscous mucous blanket lining the intestinal tract. Ferrero and Lee (10) have shown that *Campylobacter* spp. retain their motility in viscous solutions capable of immobilizing the majority of flagellated bacteria. Because the viscosity of the environment is known to cause conformational changes in the flagellar helix and so reduce the efficiency of propulsion of the flagella (38, 39), the structure of the *Campylobacter* flagellar filament appears to be particularly suited to maintaining cell motility in high viscosities.

In contrast to the simple flagella produced by most of the rod-shaped bacteria which colonize the gastrointestinal tract, the *Campylobacter* flagellar filament is complex and is composed of the flagellin products of two genes, *flaA* and *flaB* (2, 11, 13). For the best-studied example, *C. coli* VC167, the 572-residue FlaA and FlaB subunit proteins display 98% homology. These two proteins are present in the filament in significantly different amounts, however. Under standard growth procedures, FlaA is predominant, and mutants that express only the *flaA* gene product produce a filament indistinguishable in length from that of the wild-type flagellum. Mutants expressing only the *flaB* gene product produce a severely truncated filament that imparts only partial motility to the cell. The presence of both gene products in the filament is required for maximum motility.

In *C. coli* VC167, FlaA and FlaB are encoded by two tandemly oriented genes separated by 160 bp (11, 13). Northern (RNA) blot analyses have shown that the *flaA* and *flaB* genes are expressed concomitantly in wild-type cells and that the mRNA for each gene is unit length rather than polycistronic. Primer extension experiments have also confirmed the presence of single independent promoters for each gene (11). The *flaA* gene is controlled by a σ^{28} promoter (11), similar to the flagellar and chemotaxis genes of *Escherichia coli*, *Salmonella typhimurium*, and *Bacillus subtilus* (16). σ^{28} promoters are also located upstream of the flagellin genes of *Pseudomonas aeruginosa*, *Rhizobium meliloti*, *Serratia marcescens*, *Spirochaeta aurantia*, and *Treponema pallidum* (5, 15, 30, 32, 33, 42). The *flaB* gene is transcribed from a σ^{54} promoter which maps 40 bp downstream of the strong *flaA* terminator and 38 bp upstream of the *flaB* initiation codon. While this latter class of highly specialized σ^{54} promoter controls genes involved in the fixation and assimilation of nitrogen, amino acid transport components, and degradative enzymes (3, 23, 37), as well as the pilin genes of *P. aeruginosa* and *Neisseria gonorrhoeae* (17, 18, 24), the only other flagellin genes controlled by a σ^{54} promoter are those of *Caulobacter crescentus* (25, 29, 45).

Campylobacter flagella appear, therefore, to be unique, with the two structural genes encoding the filament proteins being under the control of two different classes of promoters (12, 28). Because flagellar genes controlled by σ^{54} promoters are the exception and are often subject to environmental and temporal regulation, and because the *flaB* gene product resulting from the activity of the σ^{54} promoter appears to be required for a fully functional flagellum even though it is produced at significantly lower copy number than the major FlaA filament protein (11), we undertook in this study to examine *Campylobacter flaB* σ^{54} promoter activity. To do so, we constructed a *Campylobacter flaB* σ^{54} transcriptional reporter gene fusion with chloramphenicol acetyltransferase

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(CAT) and first demonstrated that the *C. coli* VC167 *flaB* σ^{54} promoter can be utilized in *E. coli* and is subject to regulation in that host. We then recombined the construct back into the *Campylobacter* genome and showed that the *flaB* promoter is subject to environmental and growth phase-dependent regulation in its natural host. This is the first demonstration of gene regulation in *Campylobacter* spp., and here we report our findings.

MATERIALS AND METHODS

Bacterial strains, vectors, and culture conditions. *C. coli* VC167 serogroup LIO8 was originally obtained from H. Lior, National Enteric Reference Centre, Ottawa, Canada. *C. coli* VC167-T2 is a stable laboratory isolate that produces antigenic type 2 FlaA and FlaB flagellins and a full-length wild-type flagellar filament (1). The *C. coli* VC167-T2 *flaA flaB*$^+$ mutant KX15 was constructed by insertion of a kanamycin cassette into the unique *Eco*RV site of *flaA* and produces truncated FlaB flagellar filaments (2). Campylobacters were grown on Mueller-Hinton (MH) agar (Difco Laboratories, Detroit, Mich.) at 37°C in a nitrogen atmosphere containing 5% oxygen and 10% CO_2 unless stated otherwise. For assays of the time dependence of promoter activity, a biphasic MH culture system was employed. The defined media for *Campylobacter* spp. were as previously described (36), with the addition of 0.1 mM L-methionine and other supplements as described in Results.

E. coli DH5α was used as a host during cloning experiments and other plasmid DNA analyses and was routinely grown in Luria broth or on Luria agar at 37°C. Plasmid pBluescript SK was from Stratagene (La Jolla, Calif.), and pCM7 was from Pharmacia LKB (Uppsala, Sweden). pCM7 was the source of a promoterless CAT cartridge. The plasmids pILL600 and pGK2003 were as previously described (11, 21). pILL600 was the source of a *Campylobacter* kanamycin resistance gene, while pGK2003 is a mobilizable *Campylobacter* suicide vector which was constructed by cloning the origin of transfer from an IncP plasmid into the unique *Eco*RI site of pUC18.

Bacterial conjugation. Conjugal transfer from *E. coli* to *C. coli* VC167-T2 was done with donor strain *E. coli* DH5 harboring plasmid RK212.2 under the mating conditions described previously (13). Transconjugants were selected on MH agar supplemented with kanamycin (100 μg/ml) and trimethoprim (10 μg/ml) (Sigma Chemical Co., St. Louis, Mo.).

DNA manipulations. Chromosomal and plasmid DNAs were purified as previously described (2, 11, 13). Restriction and DNA-modifying enzymes and unphosphorylated *Hin*dIII linkers were from Boehringer Mannheim Biochemicals (Indianapolis, Ind.), Pharmacia LKB, or New England Biolabs (Ontario, Canada) and were used under the conditions recommended by the suppliers. For natural transformations, the biphasic method described by Wang and Taylor (44) was used. DNA sequence analysis dideoxy terminator chemistry was done with an Applied Biosystems 373A Automated DNA Sequencer (Applied Biosystems, Inc., Foster City, Calif.).

Motility testing. The *C. coli* VC167-T2 *flaA flaB*$^+$ mutant KX15 (2) was tested for motility by spotting cultures onto plates of MH (0.4% [wt/wt] agar) or defined *Campylobacter* medium (0.4% [wt/wt] agar) supplemented with 10 mM $MgSO_4$. Zones of motility were examined following incubation at 37 or 42°C for 20 h as previously described (11).

Protein electrophoresis and immunoblotting. FlaB flagellar protein was extracted from 20-h cells grown on motility plates with 0.2 M glycine (pH 2.2) as described earlier (11). Essentially standardized suspensions of bacteria were prepared in 0.2 M glycine–hydrochloride (pH 2.2) and allowed to sit at room temperature for 5 min. Bacterial cells were then removed by centrifugation at 12,000 × *g* for 3 min, and the supernatant was solubilized in sample buffer and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (15% [wt/vol] acrylamide; 150 V) in a minislab gel apparatus (Hoeffer Scientific Instruments, San Francisco, Calif.) by the method of Laemmli (22). Flagellin was then electroblotted onto nitrocellulose by the methanol Tris-glycine method of Towbin et al. (42a), and flagellin was detected by reaction with a 1:10,000 dilution of monoclonal antibody (MAb) 72c. This antibody recognizes conserved epitopes of the FlaA and FlaB flagellins of *Campylobacter* and *Helicobacter* spp. (19). The immunoreactive bands were visualized with the substrate 5-bromo-4-chloro-3-indolylphosphate (Sigma) and nitroblue tetrazolium (Sigma).

CAT assays. The amount of CAT produced was determined by the commercial CAT enzyme-linked immunosorbent assay (ELISA) (Boehringer Mannheim Biochemicals). Cell extracts (1 to 5 μg of total cell protein) were placed in microtiter wells precoated with anti-CAT antibody. During the first step, CAT contained in the cell extracts binds specifically to the coated microtiter wells. Then a digoxigenin-labeled anti-CAT antibody (anti-CAT-DIG) is bound to the immobilized CAT enzyme. During the third step, anti-CAT-DIG is detected by a peroxidase-labeled anti-DIG antibody and visualized by the following substrate reaction. The A_{405} was read with an enzyme immunoassay model ELISA reader (Biotek Instruments, Inc., Highland Park, Vt.). Purified CAT enzyme was included as the standard, and bovine serum albumin was used as an antigen control. All CAT assays were performed in duplicate with at least two separate cell extracts, and mean promoter activity was recorded as picograms of CAT per microgram of total cell protein.

RESULTS

Construction of σ^{54}-CAT fusion plasmids. To construct a transcriptional fusion between the previously mapped *flaB* σ^{54} promoter of *C. coli* VC167-T2 and the promoterless CAT reporter gene cartridge, the plasmid pBR322 was first digested with *Dra*I and then religated to remove 711 bp (692- and 19-bp fragments) between positions 3230 and 3941. This 3.65-kb plasmid was further manipulated by digestion with *Hin*dIII, end filling with Klenow fragment, and religation to generate plasmid pRIC1003. A unique feature of σ^{54}-regulated genes is the presence of an upstream activator sequence (UAS). These are usually located at least 80 bp upstream from the promoters that they regulate, although activity has been previously demonstrated when the UAS site was moved greater than 1 kb from the respective promoter (19). For this reason, the 1,119-bp *Eco*RI-*Dra*I fragment containing the 3′ end of the *flaA* gene and intergenic sequence between the two tandemly oriented *fla* genes, including the strong *flaA* transcriptional terminator which stops all transcription from the *flaA* σ^{28} promoter (13), was cloned into the unique *Eco*RI-*Dra*I sites of pRIC1003 (Fig. 1). Also contained in this *Eco*RI-*Dra*I fragment was a putative UAS (TGT-N_7-ACA) at position −100, the typical position −13-to-−26 σ^{54} consensus sequence, and other translation-transcription signals of *flaB* (GG...N_{10}...GC...N_{12}...mRNA start point...N_{15}...Shine-Dalgarno ribosomal

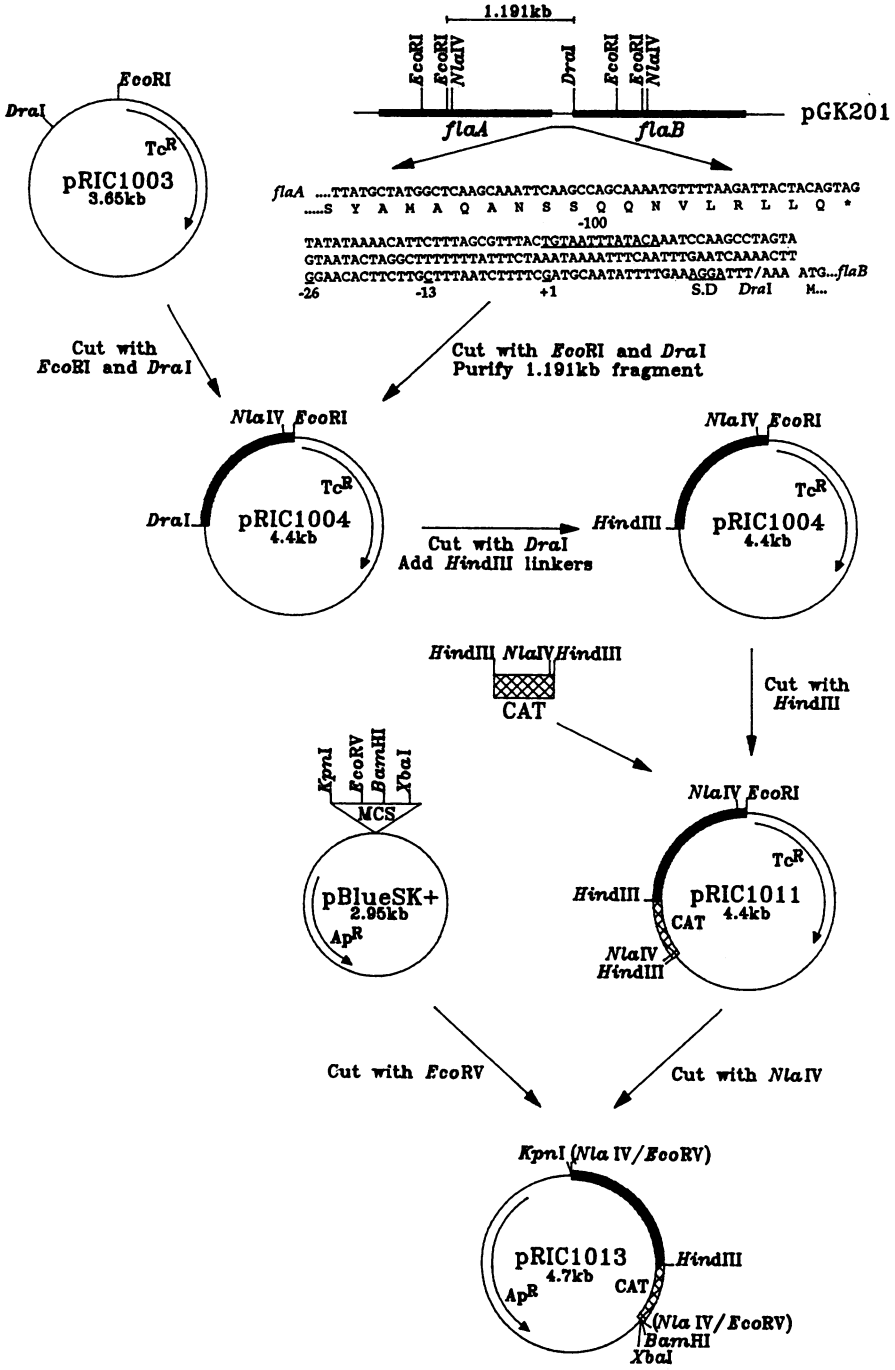

FIG. 1. Schematic representation of flagellin genes in *C. coli* VC167 and construction of plasmid pRIC1013 carrying the *flaB* σ^{54} promoter-CAT gene fusion. The 1.191-kb *Eco*RI-*Dra*I fragment indicated by the bar above the restriction sites contains the 3′ portion of the *flaA* gene, as well as the intergenic region which contains the *flaA* terminator and the transcriptional-translational regulatory sequences of *flaB*. The expanded region below shows the 3′ end of the *flaA* gene and the carboxy-terminal 19 residues of the FlaA flagellin, a putative UAS (TGT-N_7-ACA) underlined at position −100, the position −26-to-−13 consensus sequence of the *flaB* σ^{54} promoter, and the previously mapped mRNA start point (position +1). The ribosome binding site is also shown (S.D), as are the *Dra*I site, the *flaB* start codon, and the N-terminal methionine (M) residue. The σ^{54} promoter containing the *Eco*RI-*Dra*I fragment ligated into pRIC1004 and present in subsequent plasmids is indicated by the solid region, while the promoterless CAT cartridge from pCM7 is crosshatched. pBlueSK+, pBluescript II SK$^+$; MCS, multiple cloning site.

binding site) (Fig. 1). The unique *Dra*I site was converted to a *Hin*dIII site by the addition of synthetic linkers, and the promoterless CAT cartridge isolated from pCM7 was ligated in as a *Hin*dIII fragment generating pRIC1011 (Fig. 1). The *Nla*IV fragment containing the σ^{54}-CAT fusion as well as 1.1 kb of upstream DNA was cloned into the unique *Eco*RV site of pBluescript II SK$^+$, giving rise to pRIC1013 (Fig. 1). The plasmid pRIC1013 was modified by inserting the *Campylo-*

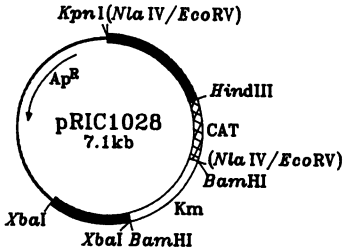

FIG. 2. Conjugative suicide plasmid pRIC1028 containing *Campylobacter* DNA (solid regions; the σ^{54} promoter is contained in the *Kpn*I-*Hin*dIII fragment), CAT cartridge (crosshatched region), and Km^r cartridge from pILL600 (open region).

bacter Km resistance cassette from pILL600 (21) as a *Bam*HI fragment downstream of the CAT gene to enable selection in *Campylobacter* spp. Further digestion with *Kpn*I and *Xba*I allowed the σ^{54}-CAT-Km^r construction to be inserted into the suicide vector pGK2003 (11), generating the conjugative plasmid pRIC1027. Finally, to direct subsequent recombination into the *Campylobacter* chromosome, a 1-kb fragment of *Campylobacter* DNA from approximately 0.8 kb downstream of *flaB* was inserted into the *Xba*I site of pRIC1027, generating pRIC1028 (Fig. 2).

Expression of the *C. coli flaB* promoter in *E. coli*. The *flaB* σ^{54} promoter from *C. coli* was functional in *E. coli* DH5α, as pRIC1013 conferred chloramphenicol resistance to this host, and the 25-kDa CAT protein was visualized in *E. coli* minicells (data not shown). To evaluate whether the activity of the *C. coli flaB* σ^{54} promoter might be affected by environmental factors in this foreign host, *E. coli* DH5α containing pRIC1013 was grown on Luria agar under varying conditions of pH, temperature, and atmosphere, and CAT gene expression from the *flaB* σ^{54} promoter was quantitated. The amount of CAT produced at pH 7.0 and 37°C was 108 pg/μg of cell protein. Figure 3 shows that maximal expression on Luria agar at 37°C was achieved at pH 9.0, with 378 pg of CAT per μg of cell protein. Because *Campylobacter* spp. are microaerophilic thermophiles, we also examined CAT expression in *E. coli* after growth in a nitrogen atmosphere containing 5% oxygen and 10% CO_2 at 37 and 42°C. Under these atmospheric conditions, the optimal pH was 8.0 at both temperatures. However, CAT expression was dramatically higher at 42°C, with the maximum measured activity at pH 8.0 being 713 pg/μg of cell protein.

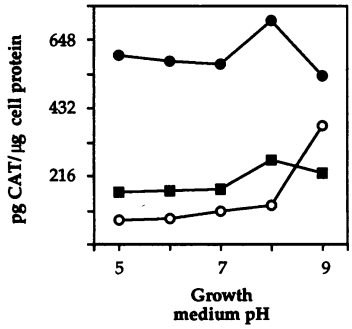

FIG. 3. Effect of growth medium pH and growth temperature on CAT expression from the *C. coli flaB* σ^{54} promoter in *E. coli*. *E. coli* DH5α containing pRIC1013 was grown for 24 h on Luria agar at various pH levels at 37°C (○) or 42°C (●) or microaerophilically at 37°C in a nitrogen atmosphere containing 5% oxygen and 10% CO_2 (■).

Expression of CAT in *C. coli* VC167-T2. These data indicated that the *C. coli flaB* σ^{54} promoter could be modulated by environmental factors. To examine regulation of this promoter in *Campylobacter* spp., the σ^{54}-CAT fusion needed to be recombined into the genome of *C. coli* VC167-T2. Plasmid pRIC1028 was conjugally mobilized from strain DH5(RK212.2) into *C. coli* VC167-T2. Transconjugants were selected on MH agar supplemented with kanamycin and trimethoprim, and under these conditions Km^r transconjugants should represent events in which the resident *flaB* gene from VC167-T2 had been replaced by the incoming fusion construct, leaving the *flaA* gene intact. Of the 24 Km^r *Campylobacter* transconjugants that were examined, all were also found to be resistant to chloramphenicol. SDS-PAGE analysis combined with motility assays indicated the presence of a full-length flagellar filament characteristic of a *flaA*$^+$ *flaB* mutant (10, 11). To confirm the σ^{54}-CAT fusion and the correct genetic linkage to *flaA*, several methods were employed. First, wild-type *C. coli* VC167-T2 was transformed with genomic DNA from a Km^r Cm^r colony (T2/28:1) by the biphasic natural transformation procedure described previously (44). A total of 126 of 126 colonies tested were resistant to both antibiotics, indicating that the cotransformation of the two markers was very tightly linked and confirming that the *E. coli* antibiotic resistance determinant was functioning in *Campylobacter* spp. Second, Southern blot hybridization to T2/28:1 DNA indicated that the suicide vector had not been incorporated into the chromosome and that *flaA* but not *flaB* was present (data not shown). Third, the entire region surrounding the flagellin genes was cloned from transconjugant VC167-T2/28-1. Sequence analysis confirmed the predicted DNA sequence through the junction between the *flaB* promoter and the CAT cartridge. Finally, polymerase chain reaction analysis confirmed that the fusion was correctly positioned in the chromosome with respect to the *flaA* gene (data not shown).

Regulation of CAT expression in *C. coli* VC167-T2/28-1. The activity of the *flaB* promoter could now be measured in *Campylobacter* spp. The effect of selected environmental factors on *flaB* promoter activity was measured with cells grown for 20 h. The reference value for CAT production from the *flaB* σ^{54} promoter in strain VC167-T2/28-1 grown under our standard *Campylobacter* reference culture conditions (37°C on MH agar [pH 7.0] in a nitrogen atmosphere containing 5% oxygen and 10% CO_2) was 132 pg of CAT per μg of total cell protein. As was the case in the *E. coli* background, *flaB* promoter activity in *Campylobacter* spp. was affected by medium pH and growth temperature (Fig. 4A), although the effects were not as pronounced in the natural host. Maximal *flaB* promoter activity of 206 pg of CAT per μg of total cell protein was obtained in cells grown at 42°C on MH (pH 8.0).

Because no minimal medium is available for *Campylobacter* spp., the previously described (36) defined medium with 0.1 mM L-methionine added was used to examine the effects of selected inorganic nutrients on *flaB* promoter expression. Figure 4B shows that in this medium *flaB* promoter expression was affected by $(NH_4)_2SO_4$ and K_2HPO_4 concentrations. With $(NH_4)_2SO_4$, maximum promoter activity of 256 pg of CAT per μg of total cell protein was obtained at 11.4 mM, declining with increasing $(NH_4)_2SO_4$. At the standard concentration of 22.96 mM K_2HPO_4 in defined medium, 162 pg of CAT per μg of total cell protein was produced, decreasing to 100 pg of CAT per μg of total cell protein at

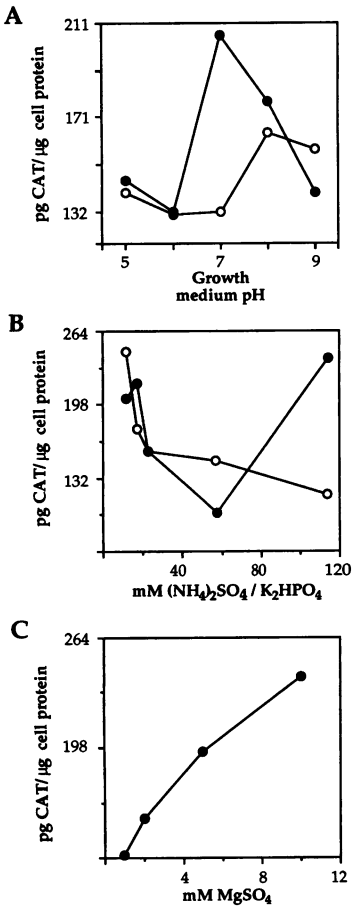

FIG. 4. Effect of various environmental factors on CAT expression from the *C. coli flaB* σ^{54} promoter in *C. coli* VC167-T2/28-1. The effects of pH and growth temperature (37°C [○] or 42°C [●]) (A), $(NH_4)_2SO_4$ (○) and K_2HPO_4 (●) (B), and $MgSO_4$ (C) were determined.

57.4 mM K_2HPO_4 and then increasing to 239 pg of CAT per μg of total cell protein at 114.8 mM K_2HPO_4. Divalent cations also affected *flaB* promoter activity. Figure 4C shows that promoter activity increased from 132 pg of CAT per μg of total cell protein at 1 mM $MgSO_4$ to 240 pg of CAT per μg of total cell protein at 10 mM $MgSO_4$. The results in Table 1

TABLE 1. Effects of divalent cations on CAT production from the *flaB* promoter in *C. coli* VC167-T2/28-1

Divalent cation and concn (mM)	CAT production (pg of CAT/μg of total cell protein)
MH agar	132
$MnSO_4$	
3.6	242
17.8	149
35.5	270
$ZnSO_4$	
2.1	116
10.4	240
20.9	128

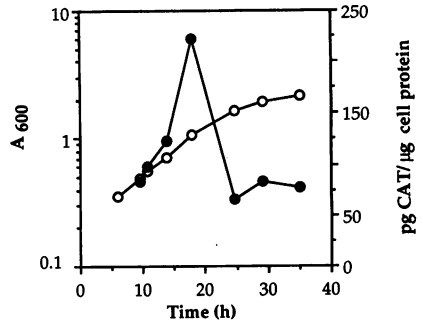

FIG. 5. Activity of the *flaB* σ^{54} promoter in *C. coli* VC167-T2/28-1 (as measured by CAT production [●]) during various phases of biphasic growth in MH broth (A_{600} [○]).

show that *flaB* promoter activity was also affected by the levels of Zn^{2+} and Mn^{2+} in the growth medium.

Because σ^{54} promoters can also be subject to temporal regulation (25, 29, 45), we also examined *C. coli flaB* σ^{54} promoter activity at different times during the growth cycle. To follow the growth of strain VC167-T2/28-1 by absorbance, cells had to be grown in the liquid phase of a biphasic MH growth system instead of MH plates; otherwise, the standard *Campylobacter* reference culture conditions were employed. Figure 5 shows that the *flaB* promoter activity varied according to the growth phase of the cells, increasing during the exponential phase of growth, peaking at mid-log phase (A_{600} = 1.0) after 18 h at 222 pg of CAT per μg of cell protein, and then declining as the culture approached stationary phase.

Effect of increased *flaB* gene transcription of FlaB production and *flaA flaB*$^+$ cell motility. Because activity of the *flaB* σ^{54} promoter could clearly be influenced by environmental factors, we examined the *flaA flaB*$^+$ mutant KX15 (11) to determine whether, as a result of environmental factors, an increase in promoter activity might be accompanied by increased synthesis of FlaB flagellar protein and increased cell motility. To evaluate the effects on FlaB flagellar protein production, the short FlaB flagellar filaments were dissociated from cells by the glycine-HCl (pH 2.2) extraction procedure and FlaB flagellin in the glycine fraction was detected by Western blot (immunoblot) analysis with the anti-*Campylobacter* flagellin MAb 72c. The first factor examined was growth temperature. We have previously reported on the poor motility conferred on *flaA flaB*$^+$ mutants (10, 11), and when *flaA flaB*$^+$ KX15 cells were grown under our standard reference culture conditions at 37°C, the cells displayed virtually no motility at 20 h on the MH motility agar (Fig. 6A, panel 1). Only a small amount of FlaB flagellin could be isolated from the cells (Fig. 6B, lane 1). Indeed, under these growth conditions, FlaB could not be detected by Coomassie blue staining, and it was barely detectable immunochemically, as is readily seen in Fig. 6B, lane 1. However, when *flaA flaB*$^+$ KX15 cells were grown at 42°C under the same conditions, they displayed greater motility (Fig. 6A, panel 2) and the quantity of FlaB flagellin recovered from these cells was noticeably increased (Fig. 6B, lane 2). We also examined the combined effects of 42°C and 10 mM $MgSO_4$ on FlaB production and the motility of *flaA flaB*$^+$ KX15 cells. This necessitated the use of the defined *Campylobacter* medium, so the results obtained were not directly comparable with those obtained with the richer MH medium. Under the combination of these two modulating

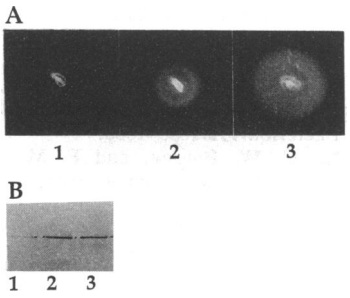

FIG. 6. (A) Motility of *C. coli* VC167 *flaA flaB*$^+$ mutant KX15 after incubation for 20 h at 37°C (panel 1) and 42°C (panel 2) on 0.4% MH agar and on *Campylobacter* defined medium containing 10 mM $MgSO_4$ at 42°C (panel 3). (B) Western blot detection of FlaB flagellin isolated from cells of *flaA flaB*$^+$ mutant KX15 after incubation for 20 h at 37°C (lane 1) and 42°C (lane 2) on 0.4% MH agar and on *Campylobacter* defined medium containing 10 mM $MgSO_4$ at 42°C (lane 3). The anti-*Campylobacter* flagellin MAb 72c was used at a 1:10,000 dilution. Equal amounts of protein were added to each lane.

factors, *flaB* σ^{54} promoter activity in VC167-T2/28-1 was elevated at 338 pg/μg of total cell protein, and both the motility exhibited by the *flaA flaB*$^+$ KX15 cells (Fig. 6A, panel 3) and the amount of FlaB isolated from these cells (Fig. 6B, lane 3) were increased over those of cells grown under our standard reference culture conditions.

DISCUSSION

The ability to modulate expression of virulence factors in response to environmental factors would appear to be a desirable attribute for pathogenic bacteria. The phenomenon has been best characterized with *Vibrio cholerae* and *Bordetella pertussis* (3), in which quite complex mechanisms operate. Campylobacters are far more common pathogens of humans than these two species, and because they are isolated from a diverse variety of habitats ranging from stream water, milk, and processed chicken meat to the gastrointestinal tracts of birds, animals, and humans (41), they might well be expected to possess mechanisms which would allow them to regulate the expression of various phenotypic properties in these different environments. The best-described virulence property of the campylobacters is their motility. Motility appears to be essential to intestinal colonization and hence to diarrheal disease (4, 7, 27), and flagella must be regarded as a virulence factor for *Campylobacter* spp. This study has provided evidence that the σ^{54} promoter of the *C. coli* gene coding for the minor FlaB flagellin which is required for maximum cell motility is susceptible to environmental modulation.

Although only a limited number of physical and inorganic chemical factors were examined, the environmental modulation of the *flaB* σ^{54} promoter activity was seen both when the promoter was carried on a plasmid in *E. coli* and when it was correctly positioned in the *Campylobacter* chromosome. While the effects were somewhat different in the two host backgrounds, *flaB* promoter activity in both species was shown to be affected by growth temperature and the pH of the growth medium. In *E. coli*, activity was also influenced by the growth atmosphere, while in *Campylobacter* spp. the levels of nitrogen, phosphate, and several divalent cations also modulated *flaB* σ^{54} promoter activity. Moreover, when cells of a *Campylobacter flaA flaB*$^+$ mutant were grown at 42°C in the presence of 10 mM $MgSO_4$, conditions which increased *flaB* σ^{54} promoter activity 2.6-fold, the *Campylobacter* cells produced increased amounts of FlaB flagellin and displayed increased motility. As such, this represents the first demonstration of environmental regulation of bacterial motility being mediated by a direct effect on the expression of a flagellar filament protein. Preliminary evidence (2a) suggests that the activity of the σ^{28} promoter of *flaA* is also increased by growth at 42°C, and not surprisingly, wild-type cells of *C. coli* VC167-T2 display greater motility at this growth temperature (12). Indeed, this ability of campylobacters to increase cell motility in response to an increase in growth temperature is probably quite important to their ability to colonize the intestinal tracts of birds and certain animals, such as dogs, which have elevated body temperatures (41).

The highest promoter activities measured in this study occurred when the *Campylobacter flaB* σ^{54} promoter was expressed off a plasmid in *E. coli* cells grown at 42°C. The reason for this high expression is unclear at this time and may simply reflect plasmid copy number. However, *E. coli* σ^{54}-RNA polymerase obviously has no trouble transcribing the *Campylobacter* σ^{54} consensus sequence. Note also that *Campylobacter* spp. similarly had no trouble transcribing and translating the *E. coli*-derived CAT gene. Indeed, this is the first successful use of a foreign resistance gene being expressed behind a *Campylobacter* promoter in the *Campylobacter* chromosome. This successful use of CAT as a reporter gene in *Campylobacter* spp. augers well for future studies of gene expression in *Campylobacter* spp., which have proven refractile to many of the genetic manipulations successfully applied to other bacteria.

The alterations in the activity of the σ^{54} promoter in *Campylobacter* spp. in response to the various individual factors examined were each quantitatively lower than in the example noted above. However, most produced changes in the twofold range, which must still be regarded as quantitatively and biologically significant. The inorganic factors modulating the changes were certainly consistent with those in other σ^{54} promoters. For example, Mg^{2+} is involved in the regulation of a temporal hemagglutinin from *Myxococcus xanthus* (20, 35). Moreover, divalent cations such as Mg^{2+}, Mn^{2+}, and Zn^{2+} appear to be important in a number of aspects related to flagella, including flagellin synthesis and flagellar assembly (34). Low oxygen tension and/or limited combined nitrogen (9, 14) has also been shown to regulate several σ^{54}-controlled *nif* and *fix* genes in certain gram-negative bacteria. In these cases, the effect is mediated by the NifA activator protein, which recognizes the UAS site TGT-N_{10}-ACA (8). Sequences of σ^{54} promoter UASs appear to differ somewhat, with TGT-N_{11}-ACA (31) and TGT-N_{10}-gCA (8) having been reported previously. Interestingly, the *Campylobacter flaB* σ^{54} promoter has a TGT-N_7-ACA site centered around the −100 position, and given the increase in transcriptional activity of the *Campylobacter flaB* σ^{54} promoter in response to nitrogen limitation, it is tempting to speculate that this −100 sequence may serve as a UAS for the *flaB* σ^{54} promoter.

The *fla* genes of *Caulobacter* spp. are also controlled by σ^{54} promoters, and in this organism this class of promoter is thought to contribute to temporal regulation of flagellar expression. *Caulobacter* cells undergo a differentiation cycle, and flagella are only produced at a specific time in this cycle (25, 29, 45). In contrast, *Campylobacter* cells do not undergo differentiation and are flagellated throughout growth. Therefore, although *flaB* expression was shown to be growth phase dependent in *Campylobacter* spp., peaking

at mid-log phase, this effect would appear not to represent true temporal regulation as exhibited by *Caulobacter* spp. Rather, the *Campylobacter flaB* effect appears to be similar to that seen in *B. subtilus*, in which the expression of flagellin gene (*hag*) transcripts also peaks during exponential growth and decreases significantly when the culture enters stationary phase (26).

In summary, campylobacters appear to have evolved a unique mechanism involving two different classes of promoters to regulate the synthesis of the two filament proteins that compose their motility organelle. The minor filament FlaB protein is controlled by a σ^{54} promoter whose expression is growth phase dependent and is subject to environmental regulation, and for a *Campylobacter flaA flaB*$^+$ mutant an increase in σ^{54} *flaB* promoter activity was shown to result in increased synthesis of FlaB protein, accompanied by an increase in cell motility. This finding suggests that campylobacters may in fact be capable of regulating their motility by varying their synthesis of alternate flagellins in response to the environment niche of the cells. Studies are therefore under way to determine the effects of environment on the expression of the σ^{28} promoter of the major FlaA flagellar filament protein.

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