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The *Escherichia coli* O157 Flagellar Regulatory Gene *flhC* and Not the Flagellin Gene *fliC* Impacts Colonization of Cattle

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A virulent European *Escherichia coli* O157:H$^-$ isolate is nonmotile due to a 12-bp deletion in the flagellar regulatory gene *flhC*. To investigate the contribution of *flhC* in the relationship between *E. coli* O157:H7 and cattle, we constructed a similar *flhC* regulatory mutant in the well-characterized strain ATCC 43894. There was no difference in the growth rate between the wild type and this regulatory mutant, but phenotypic arrays showed substrate utilization differences. Survival in the bovine gastrointestinal tract and colonization of the rectoanal junction mucosa were assessed. Mixtures of both strains were given orally or rectally to steers or administered into the rumen of cattle dually cannulated at the rumen and duodenum. One day post-oral dose, most rectal/fecal isolates (74%) were the regulatory mutant, but by 3 days post-oral dose and throughout the 42-day experiment, ≥80% of the isolates were wild type. Among steers given a rectal application of both strains, wild-type isolates were the majority of isolates recovered on all days. The regulatory mutant survived better than the wild type in both the rumen and duodenum. To test the role of motility, a filament mutant (Δ*fliC*) was constructed and similar cattle experiments were performed. On all days post-oral dose, the majority of isolates (64% to 98%) were the filament mutant. In contrast, both strains were recovered equally post-rectal application. Thus, the regulatory mutant survived passage through the bovine gastrointestinal tract better than the wild type but failed to efficiently colonize cattle, and the requirement of *flhC* for colonization was not dependent on a functional flagellum.

Shiga-toxin producing *Escherichia coli* (STEC) of the serotype O157:H7 was first recognized as an emerging human pathogen in two outbreaks of hemorrhagic colitis in 1982 (40). Since then, *E. coli* O157:H7 has been recognized as the cause of large food-borne outbreaks worldwide. This strain accounts for approximately 20,000 cases of hemorrhagic colitis annually in the United States alone (16, 27, 40) and is the predominant serotype associated with the more serious enteropathic hemolytic-uremic syndrome (HUS) that occurs in 5 to 10% of the colitis cases in North America, Asia, and Europe (6, 19, 27). In 1988, an STEC strain of the serotype O157:H$^-$, which ferments sorbitol (SF) and exhibits β-D-glucuronidase activity, was isolated from HUS patients in Bavaria, Germany, and more recently from two HUS patients in the Czech Republic (4, 14). These SF STEC O157:H$^-$ strains represent a distinct clone within the *E. coli* O157 serogroup and are associated with up to 40% of the HUS cases in Germany and central Europe (4, 12, 14). This association with the life-threatening HUS is disproportionately high compared to the total number of *E. coli* O157 serotypes associated with disease in this region and suggests that the loss of flagella may contribute to increased virulence compared to motile O157 isolates. In addition to several novel putative and defined virulence genes in these European variant strains, a 12-bp deletion in the *flhC* open reading frame (ORF) that encodes an essential phenylalanine residue was identified as the cause for the loss of flagellar synthesis (4, 14, 26).

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Motility processes in gram-negative bacteria are complex and require the coordinated transcription of more than 40 genes in 14 operons (13, 23). All genes involved in flagellum biosynthesis are transcriptionally regulated in three tiers of genes designated class I, class II, and class III. The *flhDC* operon (class I) is regulated by a variety of environmental and cell cycle signals (18, 37). Once expressed, FlhD/FlhC forms a regulatory tetramer required for transcription of class II genes. These class II genes encode the flagellar type III secretory apparatus, the hook basal/body structure, and FliA (σ^F), required for transcription of class III genes. Class III genes include the motor, chemotaxis, and flagellin or filament genes (23). While motility can be induced in many Stx-producing O157:H$^-$ strains after passage through motility medium, it cannot be restored in the European variant O157:H$^-$ strains due to the 12-bp deletion in *flhC* (26). Thus, mutations in the class I operon prevent transcription of the downstream class II and class III operons.

FlhD/FlhC has been shown to function as a global regulator of 29 operons not related to motility (29, 30); therefore, the effects of mutations in *flhDC* may not be confined to motility processes alone. Interestingly, mutations in *flhDC* have also been demonstrated for several other important pathogens, such as members of the genera *Bordetella*, *Shigella*, and *Yersinia* (1, 2, 9, 21, 38). Nonmotile members of these species contain cryptic flagellar systems, and all contain type III secretory systems as essential components of virulence (2, 15, 21, 38).

Healthy cattle are the major reservoir for the human pathogen *E. coli* O157:H7, and this serotype colonizes the bovine gastrointestinal tract (GIT) at the rectoanal junction (RAJ) mucosa (24) but is not associated with disease in ruminants (3, 11, 24, 32). Furthermore, undercooked ground beef contami-

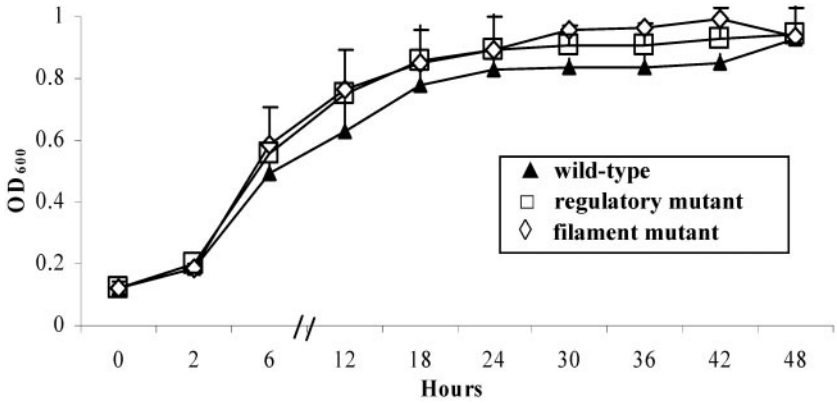


FIG. 1. Average growth of the wild type (▲), the regulatory mutant (□), and the filament mutant (◊). Cells were suspended in LB broth and incubated with aeration for the duration of the experiment. Growth was measured spectrophotometrically (optical density at 600 nm [OD_{600}]) in a Bio-Tek Power XS reader.

nated with *E. coli* O157 accounts for most human infections, causing more than half of the outbreaks in the United States alone (39). Although no reservoir host has been established for the European variant *E. coli* O157:H$^-$ strains, raw beef was implicated in a 1995 *E. coli* O157:H$^-$ HUS outbreak in Germany and a single *E. coli* O157:H$^-$ strain was isolated from a cow (14), suggesting that cattle are likely an important reservoir for the European variant *E. coli* O157:H$^-$ strains.

In light of the correlation between the loss of motility and increased pathogenesis and the fact that cattle are the major reservoir for this pathogen, we studied the effects of motility on *E. coli* O157 survival through the bovine GIT and colonization of cattle. We explored the effects of specific flagellar gene mutations in the well-characterized *E. coli* O157:H7 ATCC 43894 strain on *E. coli* O157 colonization of cattle. This particular strain of *E. coli* O157:H7, known to cause human disease, was chosen because a substantial amount of previous in vivo work demonstrates this strain behaves in a manner typical of most natural bovine carriage of the O157 serotype (10, 17, 35, 41). *E. coli* O157:H7 ATCC 43894 (referred to throughout as the wild type) was used to make an isogenic *flhC* mutant (referred to throughout as the regulatory mutant). This regulatory mutant was missing the same 12 bp identified as the *flhC* defect in the European nonmotile *E. coli* O157:H$^-$ isolate and was presumably defective in flagellar class II and class III gene expression (26). Growth rates and phenotype microarrays (PM) were used to compare the metabolic abilities of the regulatory mutant with those of the wild type. Mixtures of both strains were given orally or rectally to steers or administered directly into the rumen of cattle dually cannulated at the rumen and duodenum. Oral and ruminal administration of the bacteria were designed to assess bacterial survival in the upper bovine GIT and colonization at the RAJ mucosa. Rectal administration of the bacteria was used to assess bacterial colonization at the RAJ mucosa without the requirement of surviving passage through the GIT to arrive at this location. Standard culture techniques enumerated *E. coli* O157 in fecal, RAJ mucosa swab (RAMS), and/or digesta samples and were used to compare in vivo survival and colonization of the regulatory mutant and the wild type. To differentiate motility from other *flhC*-regulated processes, a nonmotile filament mutant (*flhC*$^+$ Δ*fliC*) defective in bacterial flagellin (referred to throughout as the filament mutant) was constructed, and similar cattle experiments were performed.

MATERIALS AND METHODS

Mutant construction and verification. The wild type, *E. coli* O157 ATCC 43894, a human stool isolate from an outbreak of hemorrhagic colitis containing genes for both Shiga toxin type 1 and type 2, was used to make the *flhC* (regulatory) and Δ*fliC* (filament) isogenic mutants. Site-directed mutagenesis was accomplished using the bacteriophage lambda recombinase system (λ-red) as described by Datsenko and Wanner (8).

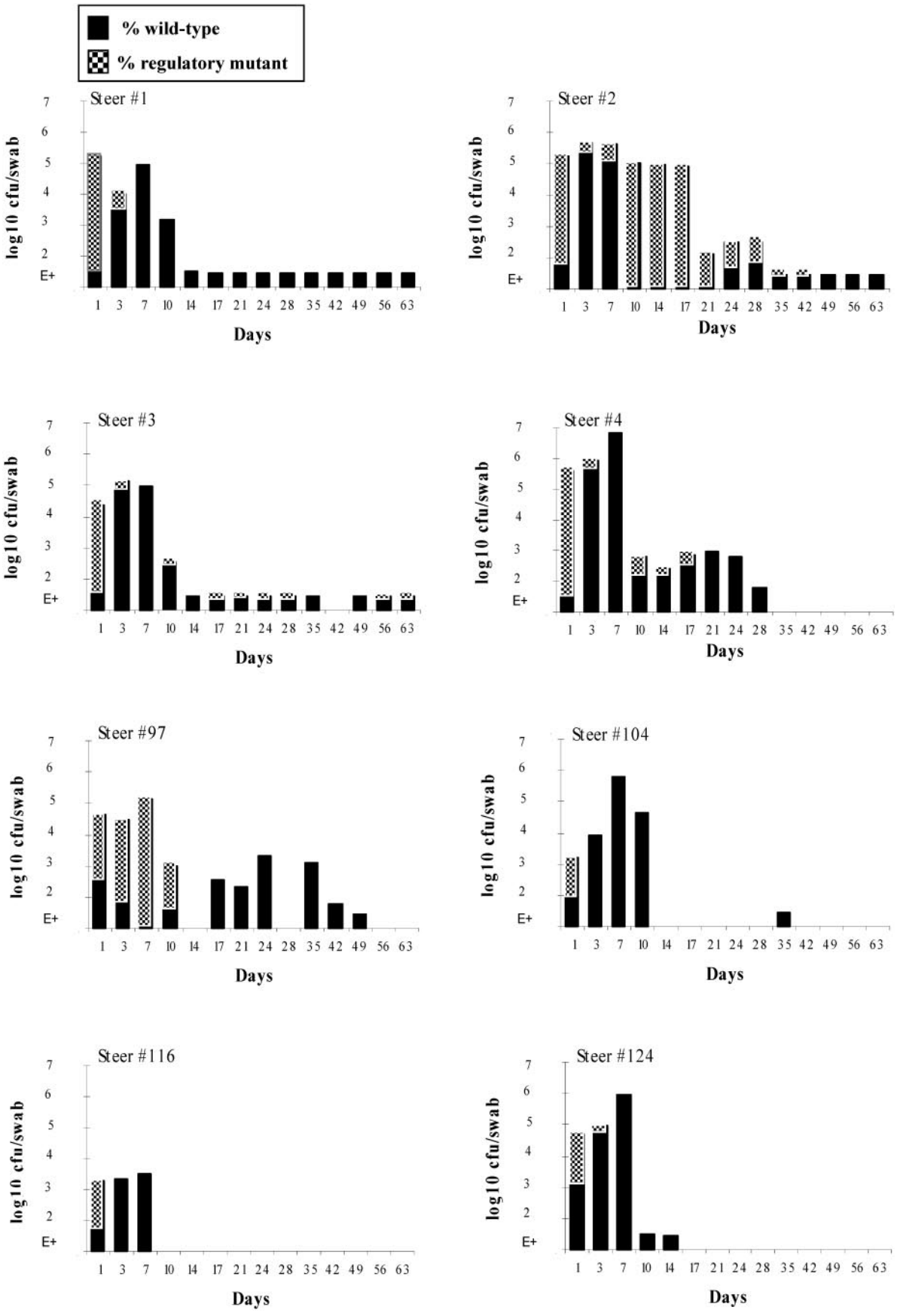
Primers for deletion were designed containing 20-bp sequences homologous to

TABLE 1. Comparison of wild-type *E. coli* O157:H7 and regulatory mutant *E. coli* O157:H$^-$ by phenotype microarray

PM substrate or chemical[a]	Difference[b]
Metabolic substrates	
Mucic acid	133
Gly-Cys	79
His-Leu	−68
Leu-B-Ala	−64
Leu-His	−60
Tyr-Ile	−58
Leu-Leu	−54
Gly-Phe-Phe	−54
Adenosine 3′,5′-cyclic monophosphate	−163
Chemical compounds	
Tolyfluanid	347
Dequalinium	144
Protamine sulfate	163
Phenylmethylsulfonyl fluoride	73
Geneticin	61
Oxytetracycline	41
2-Phenylphenol	−251
Polymyxin B	−61
Plumbagin	−62
Cephaloridine	−195

[a] Metabolic substrates or chemical compounds tested in phenotype microarray panels that were different for the regulatory mutant and the wild type.

[b] Differences are measured in arbitrary units. The average signal for a given well of the regulatory mutant or the wild type was calculated, and metabolic and chemical differences for both were expressed as the arithmetic difference of the average regulatory mutant signal minus the average wild-type signal from a given test well. Positive values indicate a greater signal, and negative values indicate a smaller signal, measured for the regulatory mutant than for the wild type.


% wild-type
% regulatory mutant
Steer #1
Steer #2
Steer #3
Steer #4
Steer #97
Steer #104
Steer #116
Steer #124
log10 cfu/swab
E+
1 3 7 10 14 17 21 24 28 35 42 49 56 63
Days

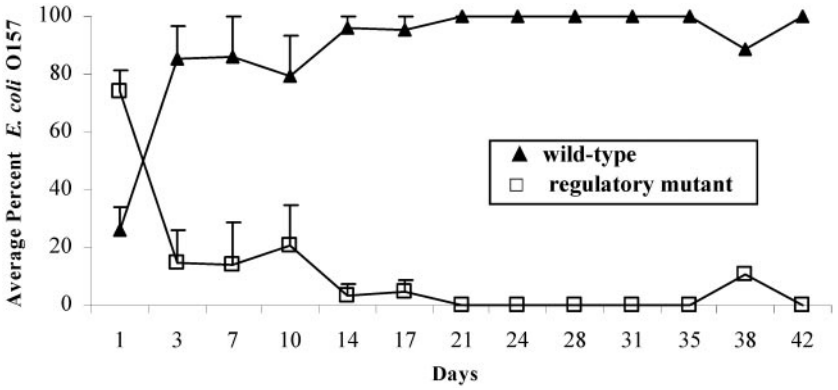


FIG. 3. Average percent motile or nonmotile *E. coli* O157 isolates at the RAJ mucosa among orally dosed cattle. Eight four- to nine-month-old Holstein steers were given a single oral dose containing 10^{10} CFU of both the wild type and the regulatory mutant on day zero. From samples positive by direct RAMS culture, 20 to 200 isolates from each steer were subcultured on motility agar, and data from steers were compiled to show the average percent motile (▲) or nonmotile (□) among seven steers.

the pKD3 template plasmid (8) and 40-bp sequences homologous to either a 12-bp region within the *E. coli* 493-89 *flhC* ORF at +374 to 385 (GenBank accession no. AY225162) as described previously (26) (forward, 5′-GACCGCT GCTGGCATTAACCCGTGCCTGGACATTGGTGCGGTGTAGGTGGAGC TGCTTC-3′; reverse, 5′-GTAATAAAATTGCCGCCGCAGCAGTTGCAGCT GGAAAGTTCATATGAATATCCTCCTTA-3′) or conserved regions within the *E. coli* Y1-57 *fliC* ORF at +157 to 1597 (forward, 5′-CGTTTTACTTCTAACAT TAAAGGCCTGACTCAGGCTGCGTGTAGGCTGGAGCTGCTTC-3′; reverse, 5′-GCCTGCTGAATGATCTGCGCTTTCGACATGTTGGACACCATATGA ATATCCTCCTTA-3′). PCR was performed using an elongase enzyme mix as recommended by Invitrogen Co. (Carlsbad, Calif.), and PCR products were verified by 0.7% agarose gel electrophoresis.

Deletions were verified by PCR as described above using primers with homologous sequences flanking either *flhC* (GenBank accession no. AE005411) (forward, 5′-GGTTGACATAAGCTGCAGGCAAAG-3′; reverse, 5′-CGGGATAT TCAGGTGGCAATGGAA-3′) or *fliC* (GenBank accession no. AY337483) (forward, 5′-GCACAAGTCATTAATACCAAC-3′; reverse, 5′-CTAACCCTG CAGCAGAGACAG-3′), sequencing on a Perkin-Elmer thermocycler using ABI Big Dye sequencing mix per the manufacturer's instructions and an ABI no. 3730 genetic analyzer. In addition, mutation was confirmed by plating cells on 0.3% tryptone agar (motility agar) and analyzing for swarming.

Bacterial growth analysis. Growth of the wild type, the regulatory mutant, and the filament mutant was analyzed using a Bio-Tek Power Wave XS reader and KCjunior software for Windows analysis. Bacteria were grown for 24 h in three ml of Luria-Bertani (LB) broth at 37°C with aeration. Twelve wells of a 96-well plate were inoculated with the wild type, the regulatory mutant, or the filament mutant in LB broth. Empty wells were filled with deionized water and the plates covered with the lid to maintain humidity and prevent spillage. All plates were incubated at 37°C and mixed at low intensity prior to every read, and optical densities were measured every five min at 600 nm for 48 h.

PM. PM were performed by Biolog Co. (Hayward, Calif.) as previously described (7). Briefly, the wild type or the regulatory mutant was grown on blood agar plates and colonies inoculated into Biolog inoculating fluid, containing a redox dye, buffer, salts, detergent, and a gelling agent that dispersed the cells evenly throughout a well. A PM panel consisted of a half-area 96-well plate such that 48 wells contained the mutant and 48 wells contained the wild type, and each well measured the function of a different enzyme, pathway, or physiological property of a cell as a change in color or turbidity. Forty PM panels representing more than 2,000 phenotypes (5) were performed for both the wild type and the regulatory mutant, and signals from individual wells were read every 15 min for the duration of the experiment by an Omnilog reader. The average signal for a given well of the regulatory mutant or the wild type was calculated, and metabolic and chemical differences for both were expressed as the arithmetic difference of the average regulatory mutant signal minus the average wild-type signal from a given test well.

Experimental cattle infection. All *E. coli* O157 cultures used for animal infection were grown in Trypticase soy broth (TSB) (Difco Laboratories, Detroit, Mich.) overnight at 37°C with aeration. Aliquots of overnight cultures were removed and serially diluted in 0.9% saline and dilutions plated on LB agar, incubated at 37°C overnight, and counted. Optical densities at 600 nm were measured, and cultures were diluted to 0.6 in sterile TSB prior administration to cattle.

(i) Oral dose. Mature Holstein steers were given a single oral dose of either 10^{10} CFU of the wild type and the regulatory mutant or 10^{10} CFU of the wild type and the filament mutant.

(ii) Rectal application. Mature Holstein steers were dosed by rectal application as previously described (35) with a mixture of bacteria containing either 10^{10} CFU of the wild type and the regulatory mutant or 10^{10} CFU of the wild type and the filament mutant. Briefly, prior to bacterial application, steers were palpated to induce defecation and the appropriate *E. coli* O157 cultures were administered rectally in a total volume of ten ml with a sterile 10-cm- by 3.5-cm-diameter cylindrical sponge (Rubbermaid, St. Francis, Wis.) inserted rectally to swab the RAJ mucosa.

(iii) Rumen application. Two mature Jersey steers dually cannulated into the rumen and the duodenum received a dose of 10^{10} CFU of both the wild type and the regulatory mutant in a total volume of ten ml into the upper rumen directly through the cannula.

Sample culture. Samples from steers orally and rectally dosed with *E. coli* O157 were cultured as previously described (32). Briefly, feces and RAMS samples were collected from each steer 24, 48, and 72 h postinoculation and twice a week thereafter into Whirl-Pak bags (Nasco, Fort Atkinson, Wis.) and three ml of TSB, respectively. All samples were placed on ice until processing in the laboratory within two h. Ten grams of feces was diluted 1:5 in TSB, and aliquots of RAMS and fecal cultures were removed, serially diluted in 0.9% saline, cultured directly (quantitative data) by plating onto sorbitol MacConkey agar supplemented with 4-methylumbelliferyl-β-D-glucuronide, cefixime, potassium tellurite, and vancomycin (SMAC-CTVM), and incubated at 37°C overnight. Subsets of sorbitol-negative, 4-methylumbelliferyl-β-D-glucuronide-negative colonies were confirmed to be *E. coli* O157 colonies by latex agglutination (Pro-Lab

FIG. 2. *E. coli* O157 colonization patterns at the RAJ mucosa among orally dosed cattle. Eight four- to nine-month-old Holstein steers were given a single oral dose containing 10^{10} CFU of both the wild type and the regulatory mutant on day zero. Colonization was assessed by direct (quantitative) and enrichment (qualitative) RAMS cultures. Bar heights represent total *E. coli* O157 CFU/swab. Samples positive only by enrichment culture had $\leq 2.8 \times 10^1$ CFU/swab and are shown as E+. No bar indicates an *E. coli* O157 culture-negative sample. For each positive sample, 20 to 100 isolates were subcultured on motility agar to determine the proportions that were mutant (checkered bar) or wild-type (filled bar) isolates recovered.

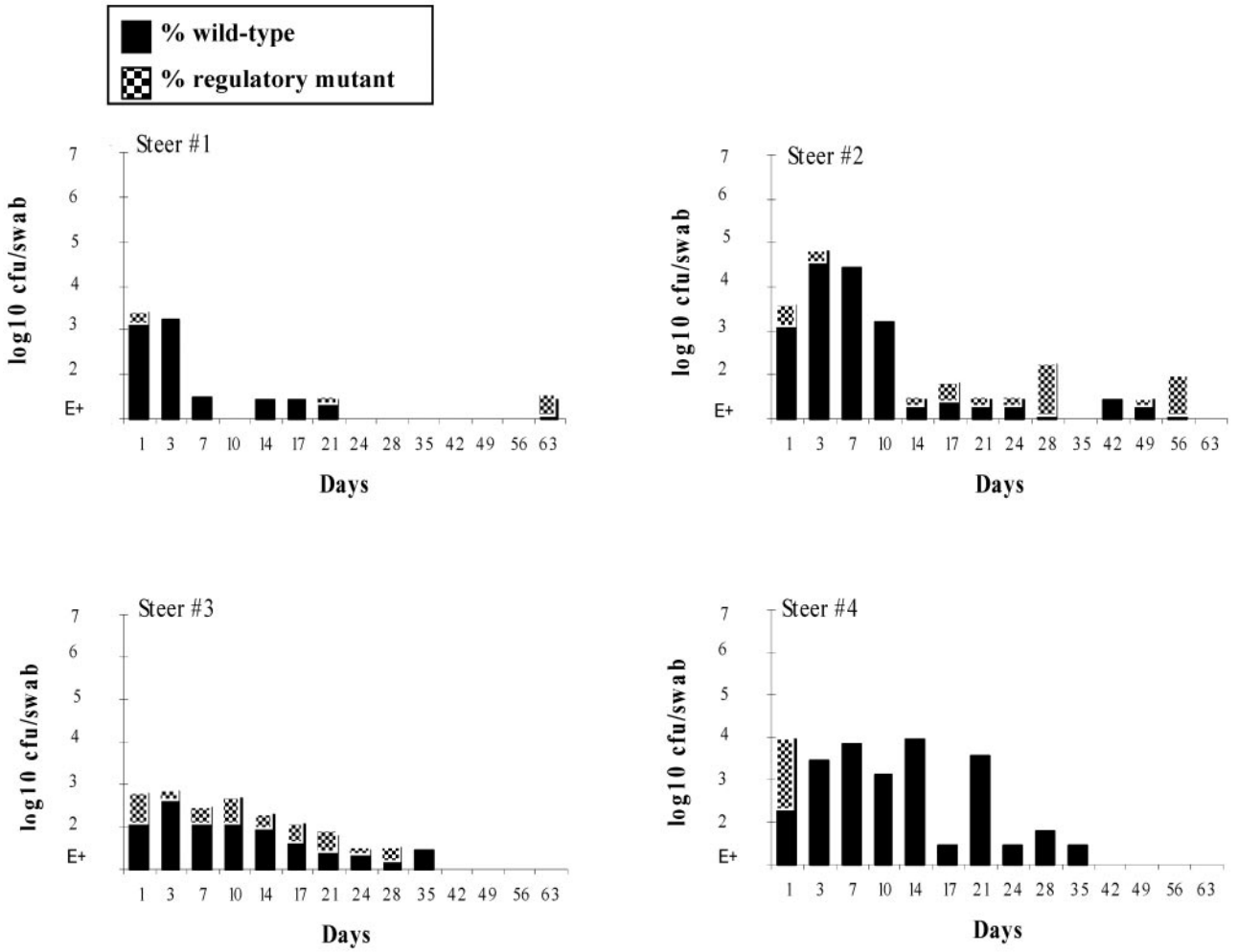


FIG. 4. *E. coli* O157 colonization patterns at the RAJ mucosa among rectally dosed cattle. Four 12- to 13-month-old Holstein steers were given a single rectal application containing 10^{10} CFU of both the wild type and the regulatory mutant on day zero. Colonization was assessed by direct (quantitative) and enrichment (qualitative) RAMS cultures. Bar heights represent total *E. coli* O157 CFU/swab. Samples positive only by enrichment culture had $\leq 2.8 \times 10^1$ CFU/swab and are shown as E+. No bar indicates an *E. coli* O157 culture-negative sample. For each positive sample, 20 to 100 isolates were subcultured on motility agar to determine the proportions that were mutant (checkered bar) or wild-type (filled bar) isolates recovered.

Diagnostics, Toronto, Canada). Samples negative by direct culture were enriched (qualitative data) at 37°C with aeration for 18 h and analyzed as described above.

Among steers inoculated directly in the rumen, digesta, feces, and RAMS samples were collected aseptically and placed on ice until processing in the laboratory within 2 h. Ten grams of rumen digesta or feces was diluted 1:5 in TSB, and 10 ml of duodenal fluid was diluted 1:5 in TSB. All samples were cultured and analyzed as described above.

Motility assays. *E. coli* O157 positive colonies from either direct or enrichment culture of digesta, feces, or RAMS were assayed for motility as previously described (36). Sterile toothpicks were used to touch the tops of isolated colonies from SMAC-CTVM plates, stabbed vertically into motility agar plates, and incubated upright at 25°C for 4 to 6 h or at 37°C for 1 to 3 h.

Statistical analysis. Repeated measures analyses of variance were conducted using SAS Proc Mixed (22) software based on the differences between the transformed proportions of the two *E. coli* O157 groups within animals. For each experiment, a set of models was fitted in Proc Mixed to select the model with the best-fitting covariance structure among the transformed differences, according to the Bayesian information criterion (34). Tests for the differences between the motile and nonmotile proportions at each time and for the interaction between the differences and the times were performed.

To calculate the transformed proportions, the proportions of motile (wild type) and nonmotile (regulatory mutant or filament mutant) groups were obtained for each animal at each time point, and if the observed proportion was either 0 or 1, then they were adjusted to $1/4n$ and $1 - 1/4n$, respectively, where $n = 20$ (28). The resulting proportions, p, were then transformed via the variance stabilizing transformation, $y = \arcsin[\text{square root } (p)]$ (28), and the differences between these transformed proportions, $y(\text{motile}) - y(\text{nonmotile})$, were used in subsequent analysis.

RESULTS

All animals in this study were culture negative for *E. coli* O157 prior to being dosed with the bacteria. The animals were healthy and did not have any ill effects from challenge with the wild type, the regulatory mutant, or the filament mutant. Furthermore, all animals were given a single oral, rectal, or ruminal dose of either the wild type and the regulatory mutant or the wild type and the filament mutant on day zero and carried *E. coli* O157 on average for 1 month, typical of experimental and natural infections with this microorganism (32). Oral and ruminal administration of the bacteria were designed to assess

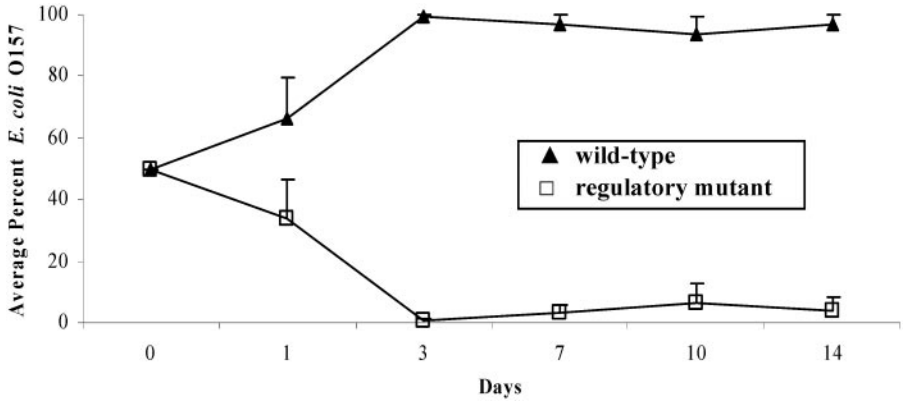


FIG. 5. Average percent motile or nonmotile *E. coli* O157 isolates at the RAJ mucosa among rectally dosed cattle. Four 12- to 13-month-old Holstein steers were given a single rectal dose containing 10^{10} CFU of both the wild type and the regulatory mutant on day zero. From samples positive by direct RAMS culture, 20 to 200 isolates from each steer were subcultured on motility agar, and data were compiled to show the average percent motile (▲) or nonmotile (□) among four steers.

the survival of each strain in the upper bovine GIT and colonization at the RAJ mucosa, while rectal administration of the bacteria was used to assess the colonization of each strain at the RAJ mucosa. Recovery of *E. coli* O157 from the ruminal digesta, duodenal digesta, feces, or RAJ mucosa was assessed by direct (quantitative) and enrichment (qualitative) culture. For each experiment, cattle expressed similar patterns of bacterial carriage of wild-type and mutant strains when bacteria were recovered from the RAJ mucosa or feces (data not shown). For each steer on each sampling day, wild-type, regulatory mutant, or filament mutant isolates were distinguished by subculture of 20 to 100 O157 isolates on motility agar. Isolates were further verified and differentiated based on PCR analysis. Wild-type and regulatory mutant strains yielded the predicted 500-bp and 564-bp products, respectively, and the wild-type and filament mutant strains yielded the predicted 1,674-bp and 318-bp products, respectively (data not shown).

Characterization of the nonmotile *E. coli* O157 mutants. Nonmotile *E. coli* O157 mutants were confirmed by sequence analysis to have either a 12-bp deletion within the *flhC* ORF +374 to 385 or a 1,441-bp deletion within the *fliC* ORF +157 to 1597 and a "scar" region of ~80 bases within the deleted region of the target genes, as previously described (8). When grown on motility agar, the wild type showed swarms of ~25 mm in diameter after 6 h at 37°C. The regulatory mutant and the filament mutant did not swarm when inoculated on motility agar. In addition, both mutants were negative for flagellin in whole-cell extracts immunoblotted with polyclonal or monoclonal antiflagellin antibody (data not shown).

Bacterial growth was indistinguishable among the wild type, the regulatory mutant, and the filament mutant, and phenotype differences were observed between the wild type and the regulatory mutant. The average growth of the wild type, the regulatory mutant, and the filament mutant was indistinguishable during all growth phases when cells were grown in LB broth at 37°C (Fig. 1).

Because *flhDC* is a global regulator of a number of nonflagellar genes (15, 29, 30), phenotypic differences between the wild type and the regulatory mutant were assessed by PM performed by Biolog Co. (Hayward, Calif.). Among the 2,000 metabolic and chemical growth phenotypes tested, 19 were different in the regulatory mutant from those in the wild type (Table 1). An empirical threshold value of 50 was used to report differences, such that a value much greater or much less than 50 indicated a larger difference between the two strains. For example, a difference of >200 was seen for 2-phenylphenol, while smaller differences were measured for several N-source di- and tripeptides containing leucine or a hydrophobic amino acid (Table 1).

Twenty-four hours after oral dosing, the regulatory mutant was recovered in larger numbers than the wild type at the RAJ mucosa, but at subsequent sampling times the wild type predominated. Oral administration was used to assess survival of the wild type and the regulatory mutant in the upper bovine GIT and colonization of both strains at the RAJ mucosa in cattle. All eight steers were culture positive 1 day after dosing, and the majority of animals remained culture positive for ≥28 days. A few steers displayed persistent carriage of the dosed bacteria through day 63 of the experiment (steers 1, 2, and 3; Fig. 2). On day 1 postinoculation, most of the *E. coli* O157 isolates recovered (59% to 96%) from seven of eight steers were the regulatory mutant (Fig. 2). However, by day 3 postinoculation, the majority of the *E. coli* O157 isolates among six of eight steers were the wild type (Fig. 2). The exceptions to this pattern were steers 2 and 7. These steers were positive by direct culture longer than any other animals in the experimental group, and the regulatory mutant was the predominant strain recovered on days 1 through 10 postinoculation (steer 97; Fig. 2) and on most days through day 28 postinoculation (steer 2; Fig. 2).

Compilation of the RAMS culture data (excluding the outlier steer, steer 2) to calculate the average percentages of the wild type and the regulatory mutant revealed a distinct pattern: day 1 postinoculation, 74% of the isolates recovered were the regulatory mutant (Fig. 3), but after this first day, the predominant and persistent strain became the wild type. The differences between wild-type and regulatory mutant proportions were significant on all days postinoculation until day 28, when only one animal remained positive by direct culture (P = 0.0403, 0.0018, 0.0014, 0.0063, 0.0033, 0.0035, 0.0040, and

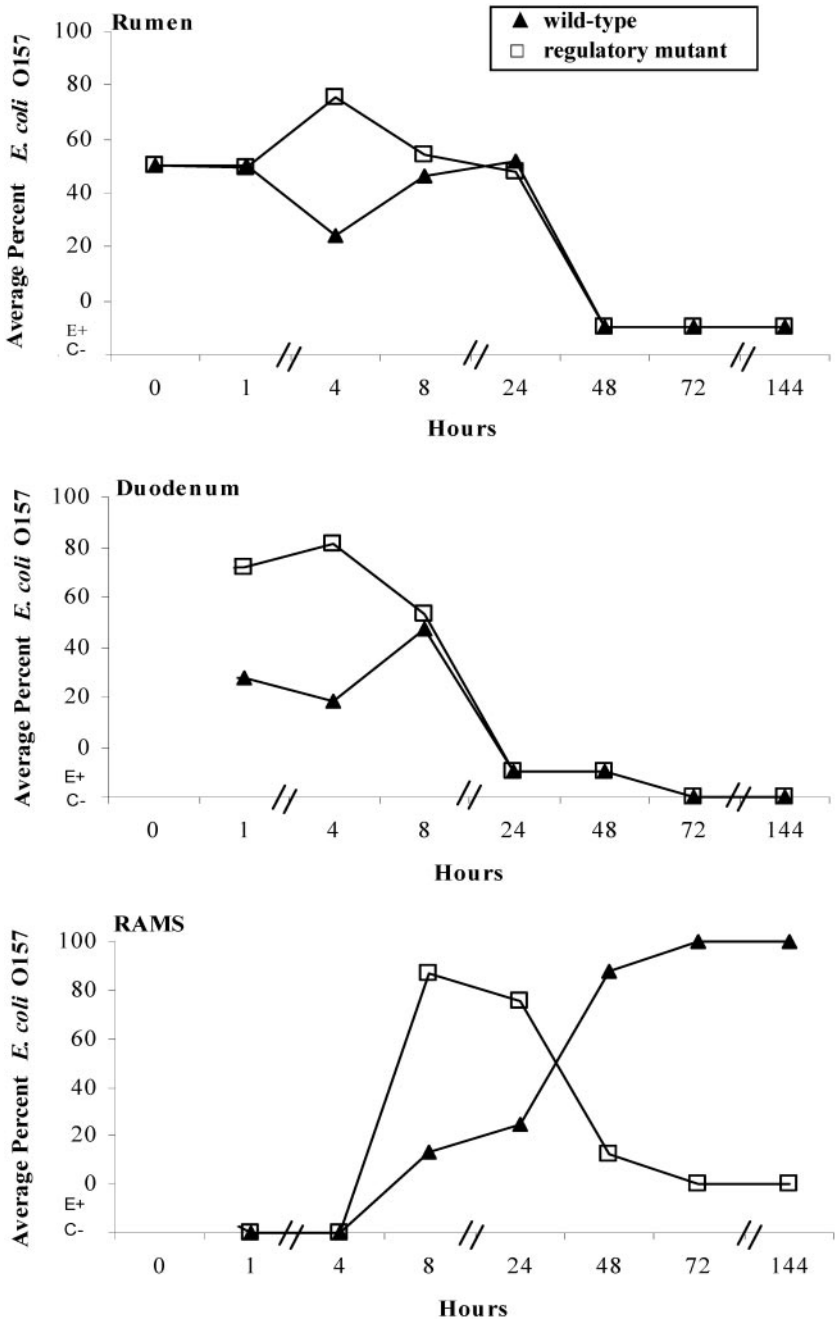


FIG. 6. Average percent motile or nonmotile *E. coli* O157 isolates from the rumen, duodenum, and RAJ mucosa of cattle given the bacteria in the rumen. Two 5-year-old Jersey steers were previously dually cannulated in the rumen and the duodenum. On day zero, animals were given a single dose containing 10^{10} CFU of both the wild type and the regulatory mutant in the upper rumen. Bacterial persistence in each compartment was assessed by direct (quantitative) and enrichment (qualitative) digesta and RAMS cultures. Ruminal or duodenal samples positive only by enrichment culture had $\leq 5.0 \times 10^1$ CFU/g, and RAMS samples positive only by enrichment culture had $\leq 2.8 \times 10^1$ CFU/swab (all shown as E+). From each sample positive by direct culture, 20 to 200 isolates from each steer were subcultured on motility agar and data were compiled as the average percent motile (▲) or nonmotile (□). *E. coli* O157 culture-negative samples are shown as C−.

0.0068, respectively; Fig. 3). The same pattern was observed when RAMS culture data from all eight animals were compiled, and the differences between wild-type and regulatory mutant proportions were significant on days 3, 7, and 21 postinoculation ($P = 0.0042$, 0.0051, and 0.003, respectively; data not shown).

Following rectal application of bacteria, the wild type was recovered in higher numbers than the regulatory mutant. Rectal administration of the bacteria was used to assess colonization of the wild type and the regulatory mutant at the RAJ mucosa in cattle. All four steers were culture positive 1 day after dose, and the majority were culture positive for ≥28 days.

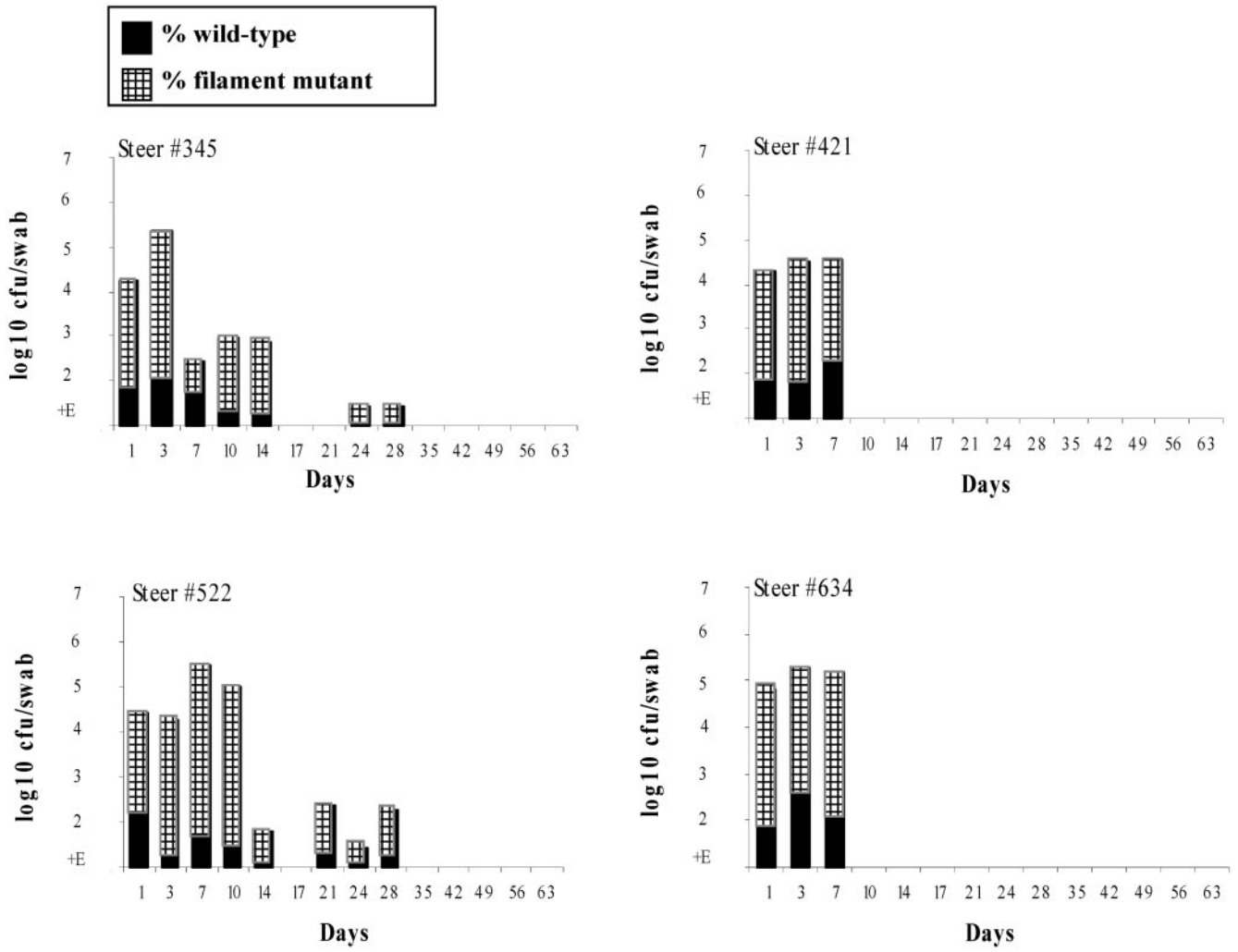


FIG. 7. *E. coli* O157 colonization patterns at the RAJ mucosa among orally dosed cattle. Four 4- to 9-month-old Holstein steers were given a single oral dose containing 10^{10} CFU of both the wild type and the filament mutant on day zero. Colonization was assessed by direct (quantitative) and enrichment (qualitative) RAMS cultures. Bar heights represent total *E. coli* O157 CFU/swab. Samples positive only by enrichment culture had $\leq 2.8 \times 10^1$ CFU/swab and are shown as E+. No bar indicates an *E. coli* O157 culture-negative sample. For each positive sample, 20 to 100 isolates were subcultured on motility agar to determine the proportions that were mutant (checkered bar) or wild-type (filled bar) isolates recovered.

One steer displayed carriage of the dosed bacteria through the 63-day duration of the experiment (steer 1; Fig. 4), and another steer displayed persistent carriage through day 56 postinoculation (steer 2; Fig. 4). On day 1 postinoculation, 57% to 94% of the *E. coli* O157 isolates recovered from three of four steers were wild type (Fig. 4), and wild-type isolates were the majority of the *E. coli* O157 isolates recovered among all four steers through the end of the 63-day experiment with few exceptions (Fig. 4).

Compilation of RAMS culture data to calculate the average percentages of the wild type and the regulatory mutant revealed a distinct pattern: the wild type was recovered more frequently than the regulatory mutant (Fig. 5). The differences between wild-type and regulatory mutant proportions were significant on days 3 through 14 postinoculation ($P = <0.0001$, 0.0001, 0.0005, and 0.0011, respectively; Fig. 5).

The regulatory mutant survived better than the wild type in the upper bovine GIT. In the previous experiments, we found that the regulatory mutant survived passage through the upper bovine GIT better than the wild type but failed to efficiently colonize the RAJ mucosa by day 3 after oral dosing in cattle given both the wild type and the regulatory mutant. Therefore, to characterize the ability of the regulatory mutant to survive in the upper GIT, we administered the wild type and the regulatory mutant in the rumen of two steers dually cannulated into the rumen and the duodenum. Both the wild type and the regulatory mutant were detected in the rumen from 1 to 144 h (day 6) postinoculation and in the duodenum from 1 to 48 h (day 2) postinoculation (Fig. 6). Steers were positive by fecal and RAMS direct culture from 8 to 144 h (day 6) postinoculation (Fig. 6) and positive by fecal and RAMS enrichment culture through the end of the experiment on day 11 postinoculation (data not shown). Although dosed with equal numbers of bacteria, the regulatory mutant was recovered in higher proportions from both the rumen and the duodenum 4 and 8 h and 1, 4, and 8 h postinoculation, respectively (Fig. 6). By 24 h postinoculation, 56% and 40% of the *E. coli* O157 isolates

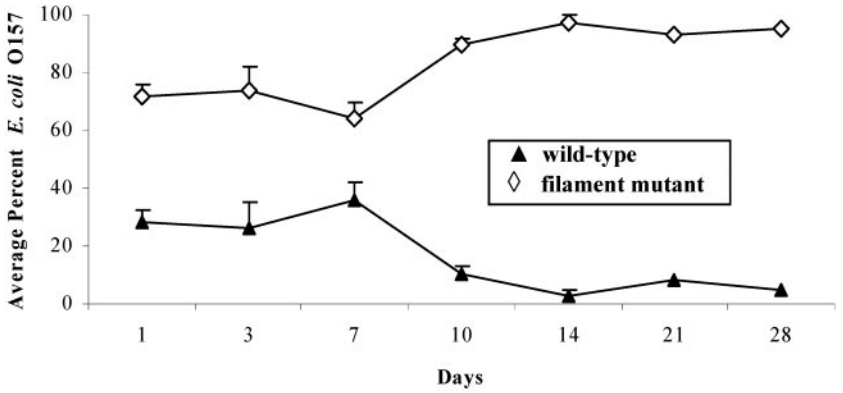


FIG. 8. Average percent motile or nonmotile *E. coli* O157 isolates at the RAJ mucosa among orally dosed cattle. Four 4- to 9-month-old Holstein steers were given a single oral dose containing 10^{10} CFU of both the wild type and the filament mutant on day zero. From samples positive by direct RAMS culture, 20 to 200 isolates from each steer were subcultured on motility agar, and data were compiled to show the average percent motile (▲) or nonmotile (◇) among four steers.

recovered from the rumen of steers 68 and 69, respectively, were the regulatory mutant (data not shown), and an average of 48% of the isolates were the regulatory mutant recovered from the rumen of both steers 24 h postinoculation (Fig. 6).

Recovery patterns of the *E. coli* O157 wild type and the regulatory mutant from RAMS and fecal (data not shown) cultures were similar to those in cattle orally inoculated with both strains (Fig. 3 and 6). At 8 and 24 h postinoculation, 87% and 76% of the *E. coli* O157 isolates recovered from RAMS direct culture were the regulatory mutant, respectively (Fig. 6). By 48 h postinoculation and through the end of the experiment, the majority of *E. coli* O157 isolates recovered in RAMS samples were the wild type (Fig. 6).

After oral dosing, the filament mutant was recovered in higher numbers than the wild type at the RAJ mucosa. To test whether the reduced colonization ability of the regulatory mutant was due to the loss of filament production, we dosed cattle with an *E. coli* O157 filament mutant. Oral administration was used to assess survival of the wild type and the filament mutant in the upper bovine GIT and colonization of both strains at the RAJ mucosa in cattle. All four steers were culture positive 1 day after dosing, and two of four steers were culture positive for 28 days. On day 1 postinoculation, 59% to 76% of the *E. coli* O157 isolates recovered from all four steers were the filament mutant (Fig. 7), and filament mutant isolates were the majority of the *E. coli* O157 isolates recovered among all steers through the end of the 63-day experiment (Fig. 7). No exceptions to this pattern were observed.

Compilation of RAMS culture data to calculate the average percentages of the wild type and the filament mutant revealed a distinct pattern: the filament mutant was recovered more often than the wild type (Fig. 8). The differences between wild-type and filament mutant proportions were significant on days 1, 3, 10, and 14 postinoculation ($P = 0.0173$, 0.0056, 0.0016, and 0.0002, respectively; Fig. 8).

Following rectal application of bacteria, the numbers of the wild type and the filament mutant recovered at the RAJ mucosa were similar. Rectal administration of the bacteria was used to assess colonization of the wild type and the filament mutant at the RAJ mucosa without passage through the bovine GIT. All four steers were culture positive 1 day after dosing, and the majority were culture positive for 24 days. One steer displayed carriage of the dosed bacteria through day 42 postinoculation (steer 401; Fig. 9). On day 1 postinoculation, 43% to 68% of the *E. coli* O157 isolates recovered from four steers were the filament mutant and 32% to 57% were the wild type (Fig. 9). No significant differences in the colonization by the filament mutant or the wild type were observed through the course of the 63-day experiment, with the exception of one steer that shed 100% filament mutant on days 17 through 28 postinoculation (steer 83; Fig. 9). When data from RAMS samples were compiled and the average percentages of the wild type and the filament mutant were analyzed, the patterns for the two strains were similar (Fig. 10).

DISCUSSION

We examined the role of flagellum biosynthesis in *E. coli* O157 colonization of the bovine reservoir by comparing wild-type *E. coli* O157:H7 strain ATCC 43894 and two nonmotile isogenic constructs: the *flhC* regulatory mutant and the Δ*fliC* filament mutant. The most important finding of this study was that both the regulatory mutant and the filament mutant had a distinct survival advantage in the upper bovine GIT compared to the wild type, but the regulatory mutant failed to efficiently colonize the bovine RAJ mucosa. The competitive disadvantage of the regulatory mutant to attach/survive at the RAJ mucosa was not due to the lack of the flagellar filament, since the filament mutant colonized the RAJ mucosa as well as the wild type. These results demonstrate the significance of the *E. coli* O157 *flhDC* operon in the colonization of the bovine host and suggest an important role of *flhDC* in functions not related to motility.

We used three methods to introduce *E. coli* O157 into cattle. The oral dose measured the ability of the bacteria to survive in and pass through the GIT, reach the RAJ mucosa, and then attach to epithelial cells in order to persist for more than a few days. The rectal application of bacteria measured only the ability of the strain to attach/survive at the RAJ mucosa and did not measure effects at other GIT locations. Application of bacteria directly into the rumen of dually cannulated steers allowed access to digesta from the rumen and the duodenum

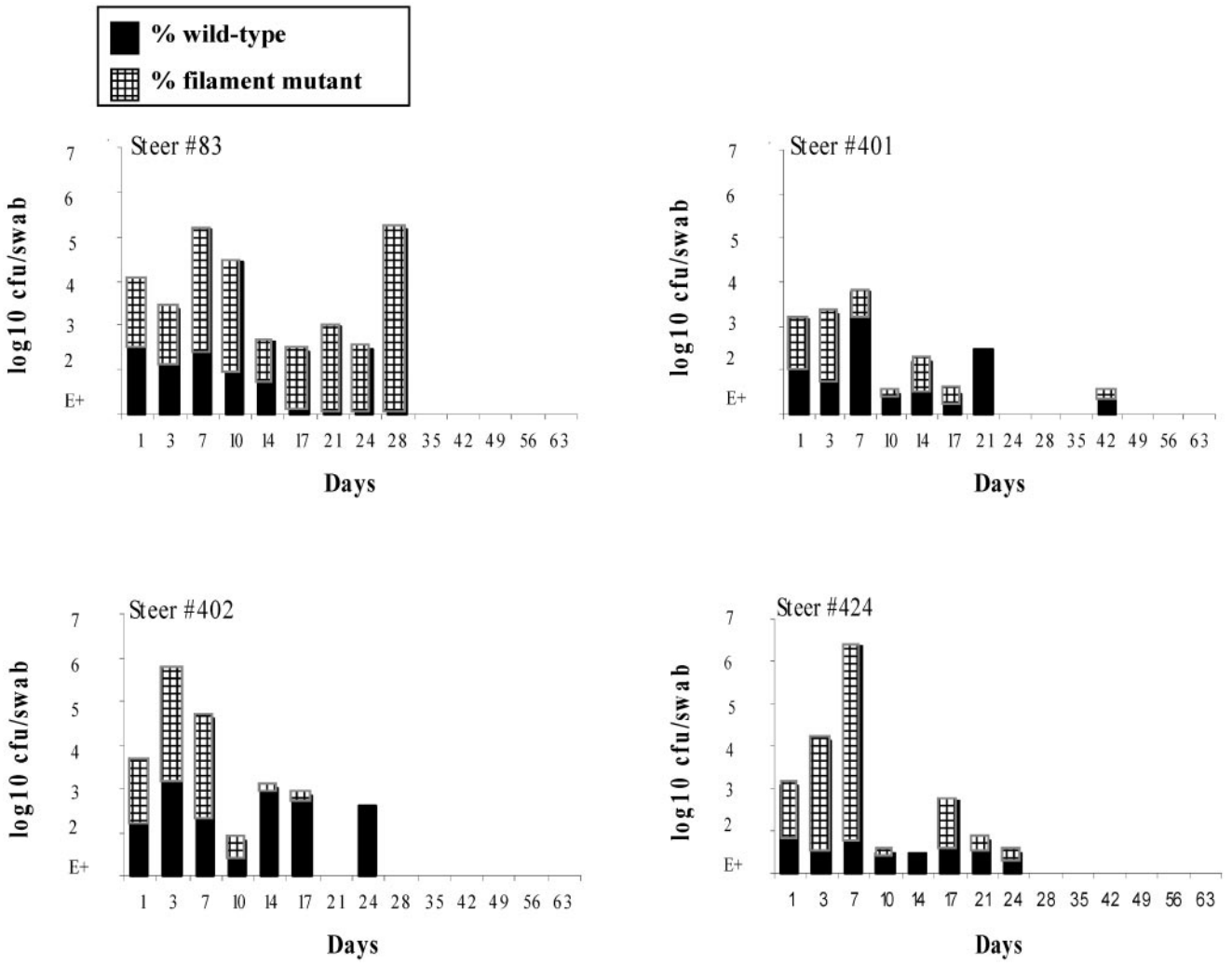


FIG. 9. *E. coli* O157 colonization patterns at the RAJ mucosa among rectally dosed cattle. Four 4- to 9-month-old Holstein steers were given a single rectal dose containing 10^{10} CFU of both the wild type and the filament mutant on day zero. Colonization was assessed by direct (quantitative) and enrichment (qualitative) RAMS cultures. Bar heights represent total *E. coli* O157 CFU/swab. Samples positive only by enrichment culture had $\leq 2.8 \times 10^1$ CFU/swab and are shown as E+. No bar indicates an *E. coli* O157 culture-negative sample. For each positive sample, 20 to 100 isolates were subcultured on motility agar to determine the proportions that were mutant (checkered bar) or wild-type (filled bar) isolates recovered.

for analysis of bacterial growth and survival in these upper GIT compartments. For the first 24 h after oral administration of bacteria, higher numbers of the regulatory or filament mutant were recovered than of the wild type. The common denominator in these experiments is that both mutants were not synthesizing flagella, resulting in significant energy conservation compared to the wild type. The filament comprises 95% of flagellum mass, and flagellar synthesis requires 2.1% of total cellular energy for production (25). A caveat to this argument is that it is not known if wild-type *E. coli* O157 synthesizes flagella in vivo. Additional explanations for the advantage displayed by these mutants may include shortened generation times, improved use of nutrients, better resistance to harsh conditions, evasion of the immune response, enhanced secretion of adhesins, and/or the ability to survive predators.

Although we did not assess all these possibilities, subtle phenotypic differences between the regulatory mutant and the wild type were revealed in a comprehensive (>2,000 substrates) Biolog PM. These metabolic differences may have reflected the better survival of the regulatory mutant in the upper bovine GIT than of the wild type. Relevant to the bovine GIT, the regulatory mutant was better able to utilize mucic acid, a component of mucus, than the wild type. Mucus bathes most of the GIT mucosa and would be a rich carbon source for bacterial growth. The PM tests are limited, however, because they are performed under a single environmental aerobic condition and are likely not a thorough measure of the various signals and metabolic differences impacting survival in the complex anaerobic ruminant GIT.

Although the regulatory mutant showed a competitive advantage in the upper GIT, it did not colonize the RAJ mucosa as well as the wild type. This suggests that the gene product(s) regulated by the FlhD/FlhC regulatory complex was required for such colonization. Recently, Leatham et al. reported that

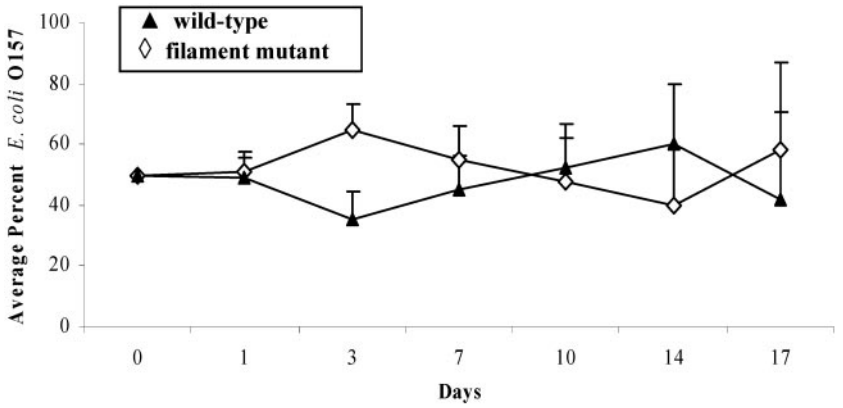


FIG. 10. Average percent motile or nonmotile *E. coli* O157 isolates at the RAJ mucosa in rectally dosed cattle. Four 4- to 9-month-old Holstein steers were given a single rectal dose containing 10^{10} CFU of both the wild type and the filament mutant on day zero. From samples positive by direct RAMS culture, 20 to 200 isolates from each steer were subcultured on motility agar, and data were compiled to show the average percent motile (▲) or nonmotile (◊) among four steers.

spontaneous *flhDC* operon mutants confer a competitive advantage to commensal *E. coli* colonizing the cecum of mice (20). When mice are fed wild-type *E. coli* MG1655, nonmotile *flhDC* operon deletion mutants are routinely isolated. One of these mutants grows up to 30% faster on several carbon sources available in the murine cecum (20). The loss of motility and improved intestinal growth of these deletion mutants are consistent with the findings we report here for cattle and provide another example of the selective advantage for some bacteria for survival in the mammalian host resulting from a class I flagellar gene mutation.

The inability of the regulatory mutant to efficiently colonize the RAJ mucosa was not due to the absence of filament for adhesion at the RAJ mucosa or chemotaxis to the site of colonization. The filament mutant appeared to colonize better than the wild type following oral dosing, but this observation may have been an artifact of higher numbers of the filament mutant generated from passage through the GIT. The filament mutant and the wild type colonized the RAJ mucosa equally, following rectal application. Although equal numbers of each strain were initially administered orally to cattle, significant differences in upper GIT survival led to significantly higher numbers of the filament mutant passing across the RAJ mucosa, and this effective "higher inoculum" favored colonization of the RAJ mucosa by the filament mutant. Therefore, while the flagellar *flhDC* master control operon is important in the colonization of the bovine host, motility, chemotaxis, or adhesive properties of the filament are surprisingly not essential for survival and persistence of *E. coli* O157 in cattle.

These observations fit a general trend emerging among a subset of important human pathogens, including *Yersinia pestis*, *Shigella* spp., *Bordetella pertussis*, and recently *E. coli*, which have lost motility by mutation in the class I *flhDC* operon; additionally, several other pathogens, such as *Yersinia enterocolitica*, *Yersinia pseudotuberculosis*, and *Bordetella bronchiseptica*, use temperature (37°C) as a key environmental cue to repress flagellum biosynthesis in the mammalian host (26). Notably, in *B. bronchiseptica* and *Salmonella enterica* serovar Typhimurium, artificial expression of flagella attenuates virulence in canine and murine models, respectively (1, 33). Thus, there appears to be a selective pressure against flagellar expression under host conditions for these organisms. Several factors may account for the repression of flagella within the host: flagellar biosynthesis and operation require 2.1% of the total cellular energy (25), flagellin is both a potent antigen and a cytokine inducer via the Toll-like 5 receptor (31), and flagellin secretion may interfere with the export of type III virulence proteins if the flagellum and type III secretion apparatuses are simultaneously expressed (42).

Our data in combination with past observations regarding the role of motility in pathogenesis may, in part, explain the increased association of the European variant *E. coli* O157:H^- strain with HUS and the failure to conclusively identify a reservoir host for these strains (4, 14). An *E. coli* O157 *flhC* regulatory mutant may be selected for within the human GIT, where it has both a growth advantage and an augmented association with mucosal epithelia due to the relief of competition between flagellar and type III virulence protein export. These effects may allow for both an increase in Shiga toxin production and morbidity in the human host. Conversely, in cattle, an *E. coli* O157 *flhC* regulatory mutant, although able to survive well as it passed through the GIT, would result in a low level of colonization at the RAJ mucosa. Thus, cattle naturally infected with *E. coli* O157 strains carrying a regulatory mutation, such as the European variant *E. coli* O157:H^- strains, may be culture positive only briefly and/or may retain bacteria at numbers below the level of conventional detection methods.

While many factors contribute to pathogenesis and colonization in the host, these results further illuminate the complexity of the host-parasite relationship and suggest a coordinated regulation of flagellum biosynthesis and pathogenic/colonization gene expression. Overall, the appearance of phenotypic variants illustrates the dynamic tension of a human pathogen with its reservoir host. Mutations conferring an advantage in one host may be a disadvantage in the other host. Experiments in this laboratory are under way to further dissect the role of *flhDC* in *E. coli* O157 colonization, including the determination of whether wild-type *E. coli* O157:H7 synthesizes flagella in the bovine host, and to analyze the role of type III secreted

proteins in the colonization of the RAJ mucosa by the regulatory and filament mutant strains described here.

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