

# Novel Repressor of *Escherichia coli* O157:H7 Motility Encoded in the Putative Fimbrial Cluster OI-1

Sarah E. Allison,<sup>a,b</sup> Uma Silphaduang,<sup>a\*</sup> Mariola Mascarenhas,<sup>c</sup> Paulina Konczyk,<sup>c</sup> Quyen Quan,<sup>c</sup> Mohamed Karmali,<sup>c</sup> and Brian K. Coombes<sup>a,b</sup>

Department of Biochemistry and Biomedical Sciences, McMaster University, Hamilton, Ontario, Canada<sup>a</sup>; Michael G. DeGroot Institute for Infectious Disease Research, Hamilton, Ontario, Canada<sup>b</sup>; and Laboratory for Foodborne Zoonoses, Public Health Agency of Canada, Guelph, Ontario, Canada<sup>c</sup>

*Escherichia coli* O157:H7 is a gastrointestinal pathogen that has become a serious public health concern, as it is associated with outbreaks and severe diseases such as hemolytic-uremic syndrome. The molecular basis of its greater virulence than that of other serotypes is not completely known. OI-1 is a putative fimbria-encoding genomic island that is found almost exclusively in O157:H7 Shiga toxin-producing *E. coli* strains and may be associated with the enhanced pathogenesis of these strains. In this study, we identified and characterized a novel repressor of flagellar synthesis encoded by OI-1. We showed that deletion of Z0021 increased the motility of *E. coli* O157:H7, which correlated with an increase in flagellin production and enhanced assembly of flagella on the cell surface. In contrast, overexpression of Z0021 inhibited motility. We demonstrated that Z0021 exerted its regulatory effects downstream of the transcription and translation of *flhDC* but prior to the activation of class II/III promoters. Furthermore, the master regulator of flagellar synthesis, FlhD<sub>4</sub>C<sub>2</sub>, was shown to be a high-copy suppressor of the nonmotile phenotype associated with elevated levels of Z0021—a finding consistent with Z0021-FlhD<sub>4</sub>C<sub>2</sub> being a potential regulatory complex. This work provides insight into the mechanism by which Z0021, which we have named *fmrA*, represses flagellar synthesis and is the first report of a fimbrial-operon-encoded inhibitor of motility in *E. coli* O157:H7.

Shiga toxin-producing *Escherichia coli* (STEC) is a zoonotic food-borne and waterborne pathogen that has been implicated in outbreaks worldwide and can cause hemorrhagic colitis and the potentially fatal hemolytic-uremic syndrome (7, 14, 16, 17, 24). The O157:H7 serotype of enterohemorrhagic STEC (EHEC), classified as a seropathotype A strain, is one of the most prevalent and is recognized for its high virulence in human populations (10, 13, 24). A genomic comparison of *E. coli* O157:H7 with serotype O26:H11, which is less frequently associated with human outbreaks and disease, revealed the presence of four fimbria-encoding genomic islands unique to seropathotype A strains: O-island 1 (OI-1), OI-47, OI-141, and OI-154 (30). Although the mechanisms underlying the unique pathogenesis of *E. coli* O157:H7 compared to that of other STEC strains are not fully known, these four OIs may be associated with the ability of O157:H7 to colonize humans and cause disease more readily than other serotypes do (30).

Fimbria-mediated adherence of *E. coli* O157:H7 to intestinal epithelial cells is an important and early step in the colonization process. The sequencing of two *E. coli* O157:H7 outbreak strains revealed the presence of at least 16 fimbrial operons, many of the chaperone/usher class (12, 26). Of the four fimbria-encoding OIs that are present in seropathotype A strains, two have been subjects of investigation. OI-141 and OI-154 encode long polar fimbriae (LPF), which were first described in *Salmonella enterica* serovar Typhimurium and show similarity to type I fimbriae (2). Torres and colleagues showed that the LPF1 cluster of *E. coli* O157:H7 located in OI-141 increases fimbrial expression and adherence to tissue culture cells when introduced into a nonfimbriated *E. coli* K-12 strain (35). Furthermore, the LPF1 cluster has a demonstrated role in adherence to epithelial cells and microcolony formation (35). The second *lpf* gene cluster of *E. coli* O157:H7, located in OI-154, is implicated in the initial stages of adhesion, and a similar region in *E. coli* O113:H21 has been shown to mediate

adherence to epithelial cells (9, 36). In contrast to OI-141 and OI-154, little is known about OI-1 and OI-47. The major fimbrial proteins encoded by OI-47 are also related to *Salmonella* LPF, and this OI is predicted to contain additional putative virulence genes (30). OI-1 is distinct from these three LPF-related clusters and is predicted to encode type 1-like fimbriae. The putative fimbrial genes in OI-1 are present in an order highly conserved with respect to that of the *E. coli* type 1 fimbrial locus (30) (Fig. 1).

In addition to fimbriae, flagella also play an important role in *E. coli* O157:H7 pathogenesis, as they enable the bacteria to breach the intestinal mucus layer to access the intestinal epithelium. Flagellar synthesis is a tightly regulated and highly energetic three-tier process (21). The early genes *flhDC* encode the master regulator FlhD<sub>4</sub>C<sub>2</sub>, which is required for the transcription of the middle genes from class II promoters (5). The middle genes encode the structural and assembly proteins required for the synthesis of the hook-basal body, the alternative sigma factor FliA ( $\sigma$ 28), and its anti-sigma factor FlgM (anti- $\sigma$ 28). Once the hook-basal body has been assembled, FlgM is secreted, thereby allowing FliA to activate the transcription of the late genes that encode flagellin and the motor and chemotaxis proteins (4). Given that adherence and motility represent antagonistic functions, bacteria use mechanisms to reciprocally regulate these processes. For instance, the

Received 8 June 2012 Accepted 24 July 2012

Published ahead of print 27 July 2012

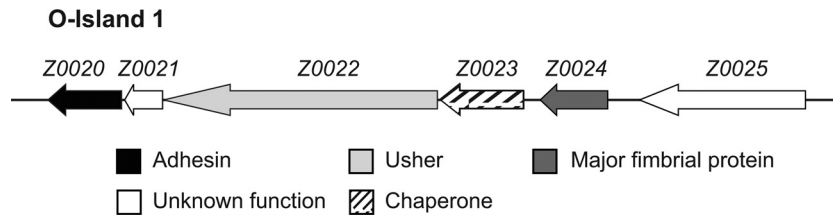
Address correspondence to Brian K. Coombes, coombes@mcmaster.ca.

\* Present address: Uma Silphaduang, Laboratory for Foodborne Zoonoses, Public Health Agency of Canada, Guelph, Ontario, Canada.

S.E.A. and U.S. contributed equally to this work.

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doi:10.1128/JB.01025-12



**FIG 1** Genetic organization of OI-1 in *E. coli* O157:H7 strain EDL933. Arrows represent open reading frames, and the putative functions of the open reading frames based on BLAST similarity are shown.

BvgAS two-component signal transduction system in *Bordetella pertussis* activates adhesin genes while repressing those involved in motility (1). There are also proteins encoded within fimbrial operons that have been shown to regulate motility. In *Proteus mirabilis*, MrpJ, which is encoded by the MR/P fimbrial gene cluster, inhibits swimming and swarming motility by repressing the transcription of *flhDC* (19). The functional homologue of MrpJ in uropathogenic *E. coli*, PapX, has been shown to repress motility by binding directly to the *flhDC* promoter (27, 31). To date, there have been no fimbrial-operon-encoded regulators of motility identified in *E. coli* O157:H7.

We turned our attention to the OI-1 genomic island as a potential source of virulence determinants associated with O157:H7 EHEC. In this work, we identified and characterized a novel repressor of motility in *E. coli* O157:H7, Z0021, encoded by OI-1. We show that deletion of Z0021 leads to greater swimming motility and flagellar production in *E. coli* O157:H7 than in the parental strain while overexpression of Z0021 inhibits motility. Using a transcriptional reporter system, we demonstrate that Z0021 regulates motility prior to middle and late gene transcription but downstream of the master regulator, FlhD<sub>4</sub>C<sub>2</sub>. In addition, we show that FlhD<sub>4</sub>C<sub>2</sub> is a high-copy suppressor of the nonmotile phenotype associated with the overexpression of Z0021. In keeping with this functional role, we propose that Z0021 be renamed *fmrA* (fimbrial-operon-encoded motility regulator A). This paper reports on a novel fimbrial-operon-encoded regulator of flagellar synthesis in *E. coli* O157:H7 and provides insight into the mechanism by which Z0021/FmrA represses motility.

## MATERIALS AND METHODS

**General methods.** Table 1 lists the strains, plasmids, and oligonucleotides used in this work. *E. coli* strains were grown in Luria broth (LB), and when necessary, antibiotics were used at the following concentrations: ampicillin, 100 μg/ml; kanamycin, 50 μg/ml; chloramphenicol, 34 μg/ml. Gel extraction and plasmid miniprep kits were purchased from Qiagen (Mississauga, Ontario, Canada). Vent polymerase was obtained from New England Biolabs (Beverly, MA), and restriction enzymes were purchased from Fermentas (Burlington, Ontario, Canada).

**Construction of mutants and HA-tagged allelic variants.** The deletion of single or multiple genes within OI-1 was carried out as described by Datsenko and Wanner (8). Linear DNA was PCR amplified from pKD4 by using the primers listed in Table 1. *E. coli* O157:H7 strain EDL933 harboring pKD46 was transformed with the concentrated PCR product, plated onto LB supplemented with kanamycin, and incubated overnight at 37°C. All of the strains generated were confirmed by PCR. A similar strategy using pSU315 (37) was employed to create strains in which *flhC* had been tagged with tandem hemagglutinin (HA) epitopes at the carboxy terminus.

**Motility assays.** Swimming motility was assessed by using 0.25% LB agar plates. Overnight cultures were standardized to an optical density at

600 nm (OD<sub>600</sub>) of 1.0, and 2 μl of culture was stabbed into the agar plates by using a sterile pipette tip. Ampicillin was added to the plates for maintenance of plasmids, and isopropyl-β-D-thiogalactopyranoside (IPTG) and arabinose were added to the plates for induction when necessary. The plates were incubated for 6 h at 37°C, after which time the diameter of the swimming zone around the inoculation site was measured.

**TEM.** Wild-type EDL933 and ΔOI-1 and ΔZ0021 mutant strains were cultured on motility agar for 6 h at 37°C. Bacteria were adsorbed to carbon-stabilized Formvar supports on 200-mesh copper transmission electron microscopy (TEM) grids by floating the grids with the Formvar side down on a drop of culture for 30 s and then rapidly washing them with water. Bacteria on TEM grids were stained by submerging the grids for 10 s in 0.1% (wt/vol) uranyl acetate and then examined with a Philips CM10 transmission electron microscope at an operating voltage at 80 kV. Digital images of bacteria were captured with an Olympus Soft Imaging Systems Morada 11-megapixel charge-coupled device camera (Biophysics Interdepartment Group, University of Guelph, Guelph, Ontario, Canada).

**Western blot analysis.** To examine the levels of H7 flagellin in the wild-type EDL933 and ΔOI-1 and ΔZ0021 mutant strains, cultures were grown in LB for 3, 6, or 24 h at 37°C. Whole-cell lysates were resolved on a 10% sodium dodecyl sulfate-polyacrylamide gel and transferred to a polyvinylidene difluoride membrane (Immobilon-P; Millipore Corp.). The blots were incubated with a 1:2,000 dilution of rabbit polyclonal antiserum to H7 flagellin, followed by a 1:2,000 dilution of peroxidase-conjugated mouse anti-rabbit immunoglobulin G, and developed using a chemiluminescence detection system (Pierce Chemical Company, Rockford, IL). For Western blot analysis of FlhC-HA in the wild-type EDL933 and ΔZ0021 mutant strains, cultures were grown in LB at 37°C to an OD<sub>600</sub> of ~0.5. Whole-cell lysates were collected and probed using anti-HA (1:2,000) and anti-DnaK (1:10,000) antibodies. DnaK served as a loading control.

**Transcriptional reporter assays.** Luciferase reporter constructs for class I, II, and III promoters were generated by PCR amplification of promoter regions from *E. coli* O157:H7 strain EDL933 genomic DNA by using primers listed in Table 1. The PCR products were cloned into pCS26 (3) and transformed into wild-type EDL933 and ΔZ0021 mutant strains. The sequences of all constructs were confirmed by sequencing. Overnight cultures were subcultured into LB to a starting OD<sub>600</sub> of 0.005 and grown for 6 h with shaking. Luminescence of cultures was measured directly (EnVision; Perkin-Elmer), and output was relative light units normalized to the OD<sub>600</sub>. All experiments were performed in triplicate.

**Quantitative real-time PCR (qRT-PCR) analysis.** Wild-type and ΔZ0021 mutant strains were grown in LB at 37°C to an OD<sub>600</sub> of ~0.6, and total RNA was isolated using a High Pure RNA isolation kit (Roche, Laval, Quebec, Canada). RNA was treated with DNase I (Ambion TURBO DNA-free kit, Applied Biosystems, Foster City, CA). First-strand cDNA was synthesized from 500 ng total RNA by using the Transcriptor First Strand cDNA synthesis kit (Roche). RT-PCR amplification was performed with 5 μl cDNA in a reaction mixture containing 1× LightCycler 480 CYBR Green I Master (Roche) and the forward and reverse primers at 500 nM (each) in a 20-μl volume. Relative quantification was performed using the LightCycler 480 Relative Quantification software (Roche) to compare the relative expression of selected gene targets to that of the *icdA*

TABLE 1 Strains, plasmids, and oligonucleotides used in this study

Strain, plasmid, or oligonucleotide	Description <sup>a</sup>	Reference or source
<b>Strains</b>		
BKC15-1	EHEC serotype O157:H7, wild-type strain EDL933	26
BKC19-79	EDL933 with deletion of OI-1 ( $\Delta$ OI-1::kan)	This study
BKC19-81	EDL933 $\Delta$ Z0020; Kan <sup>r</sup>	This study
BKC20-1	EDL933 $\Delta$ Z0021; Kan <sup>r</sup>	This study
BKC15-3	EDL933 $\Delta$ Z0021; kan cassette excised	This study
BKC20-7	EDL933 $\Delta$ Z0022 $\Delta$ Z0023 $\Delta$ Z0024 $\Delta$ Z0025; Kan <sup>r</sup>	This study
BKC30-74	EDL93/pCS26- <i>flhD</i>	This study
BKC30-75	EDL93/pCS26- <i>fliE</i>	This study
BKC30-76	EDL93/pCS26- <i>fliL</i>	This study
BKC30-77	EDL93/pCS26- <i>flhB</i>	This study
BKC30-78	EDL93/pCS26- <i>flgB</i>	This study
BKC30-79	EDL93/pCS26- <i>flgA</i>	This study
BKC30-80	EDL93/pCS26- <i>fliF</i>	This study
BKC30-81	EDL93/pCS26- <i>fliA</i>	This study
BKC31-1	EDL93/pCS26- <i>fliC</i>	This study
BKC31-2	EDL93/pCS26- <i>motA</i>	This study
BKC31-3	EDL933 $\Delta$ Z0021 (BKC15-3)/pCS26- <i>flhD</i>	This study
BKC31-4	EDL933 $\Delta$ Z0021 (BKC15-3)/pCS26- <i>fliE</i>	This study
BKC31-5	EDL933 $\Delta$ Z0021 (BKC15-3)/pCS26- <i>fliL</i>	This study
BKC31-6	EDL933 $\Delta$ Z0021 (BKC15-3)/pCS26- <i>flhB</i>	This study
BKC31-7	EDL933 $\Delta$ Z0021 (BKC15-3)/pCS26- <i>flgB</i>	This study
BKC31-8	EDL933 $\Delta$ Z0021 (BKC15-3)/pCS26- <i>flgA</i>	This study
BKC31-9	EDL933 $\Delta$ Z0021 (BKC15-3)/pCS26- <i>fliF</i>	This study
BKC31-10	EDL933 $\Delta$ Z0021 (BKC15-3)/pCS26- <i>fliA</i>	This study
BKC31-11	EDL933 $\Delta$ Z0021 (BKC15-3)/pCS26- <i>fliC</i>	This study
BKC31-12	EDL933 $\Delta$ Z0021 (BKC15-3)/pCS26- <i>motA</i>	This study
BKC16-15	EDL933 <i>flhD</i> :: <i>flhD</i> -HA	This study
BKC16-16	EDL933 <i>flhC</i> :: <i>flhC</i> -HA	This study
BKC31-14	EDL933 $\Delta$ Z0021 <i>flhC</i> :: <i>flhC</i> -HA	This study
BKC31-15	EDL933/pFLAG-CTC	This study
BKC31-16	EDL933/pFLAG-Z0021	This study
BKC31-17	EDL933/pFLAG-Z0021- <i>araC</i> -P <sub>BAD</sub>	This study
BKC31-18	EDL933/pFLAG-Z0021- <i>flhDC</i>	This study
BKC1-14	DH5 $\alpha$ ( <i>hsdR recA lacZYA</i> $\phi$ 80 <i>lacZ</i> $\Delta$ M15)	Lab collection
<b>Plasmids</b>		
pKD4	oriRy; Kan <sup>r</sup> cassette flanked by FRT sites	8
pSU315	HA epitope sequence with Kan <sup>r</sup> cassette flanked by FRT sites	37
pKD46	RepA1019(Ts), $\lambda$ , $\gamma$ , $\beta$ , and <i>exo</i> expressed from <i>ParaBAD</i> ; Amp <sup>r</sup>	8
pCS26	pSC101 <i>ori</i> , <i>luxCDABE</i> ; Kan <sup>r</sup>	3
pCS26- <i>flhD</i>	<i>flhD</i> promoter from EDL933 fused to <i>luxCDABE</i> in pCS26; Kan <sup>r</sup>	This study
pCS26- <i>fliE</i>	<i>fliE</i> promoter from EDL933 fused to <i>luxCDABE</i> in pCS26; Kan <sup>r</sup>	This study
pCS26- <i>fliL</i>	<i>fliL</i> promoter from EDL933 fused to <i>luxCDABE</i> in pCS26; Kan <sup>r</sup>	This study
pCS26- <i>flhB</i>	<i>flhB</i> promoter from EDL933 fused to <i>luxCDABE</i> in pCS26; Kan <sup>r</sup>	This study
pCS26- <i>flgB</i>	<i>flgB</i> promoter from EDL933 fused to <i>luxCDABE</i> in pCS26; Kan <sup>r</sup>	This study
pCS26- <i>flgA</i>	<i>flgA</i> promoter from EDL933 fused to <i>luxCDABE</i> in pCS26; Kan <sup>r</sup>	This study
pCS26- <i>fliF</i>	<i>fliF</i> promoter from EDL933 fused to <i>luxCDABE</i> in pCS26; Kan <sup>r</sup>	This study
pCS26- <i>fliA</i>	<i>fliA</i> promoter from EDL933 fused to <i>luxCDABE</i> in pCS26; Kan <sup>r</sup>	This study
pCS26- <i>fliC</i>	<i>fliC</i> promoter from EDL933 fused to <i>luxCDABE</i> in pCS26; Kan <sup>r</sup>	This study
pCS26- <i>motA</i>	<i>motA</i> promoter from EDL933 fused to <i>luxCDABE</i> in pCS26; Kan <sup>r</sup>	This study
pFLAG-CTC	Cytoplasmic expression of C-terminal FLAG fusion protein under control of <i>Ptac</i> ; Amp <sup>r</sup>	Sigma
pFLAG-Z0021	pFLAG-CTC carrying Z0021 with native stop codon; Amp <sup>r</sup>	This study
pBAD33	Arabinose-inducible expression vector; Cm <sup>r</sup>	11
pFLAG-Z0021- <i>araC</i> -P <sub>BAD</sub>	pFLAG-CTC carrying Z0021 with native stop codon at HindIII/KpnI sites and <i>araC</i> -P <sub>BAD</sub> cloned in at BglIII/SalI sites; Amp <sup>r</sup>	This study
pFLAG-Z0021- <i>flhDC</i>	pFLAG-CTC carrying Z0021 with native stop codon and <i>araC</i> -P <sub>BAD</sub> - <i>flhDC</i> cloned in at BglIII/SalI sites; Amp <sup>r</sup>	This study
<b>Primers</b>		
Red-OI-1-F	GGAATGGTGAAATTTATCGCAGATAGCATTCTCTCTAAATTTATAAGATGTGTAGGCTGGAGCT GCTTCG	

(Continued on following page)

TABLE 1 (Continued)

Strain, plasmid, or oligonucleotide	Description <sup>a</sup>	Reference or source
Red-OI-1-R	CTCATAAATTAAATTAATAAAAAATTCATCAACTTCAAGGACTGATAATATGAATGTCCTCCTTA	
Red-Z0020-F	GCGCTTCCAGGGAAGTCATCTTATAATTTAGAAGAAGTGTAGGCTGGAGCTGCTTCG	
Red-Z0020-R	CGGCAGCTCCCCCAAAGTTAAGGTGGGGGAGATAGACATATGAATATCCTCCTTA	
Red-Z0021-F	CGATGAAAACAAAACATATATATGCCAGTAAAAGGAGTTTACTGTGTAGGCTGGAGCTGCTTCG	
Red-Z0021-R	GTATGGAATGGTGAAATTTATCGCAGATAGCATTCTTCTAAACATATGAATATCCTCCTTA	
Red-Z0022-F	CATAGTAAAATCGCCGCGAGATAACAGGAAAAAGTCGTGTAGGCTGGAGCTGCTTCG	
Red-Z0022-R	TGTGATTAATAAAAAGCCACTTCATAGTAAACTCCTTCATATGAATATCCTCCTTA	
Red-Z0022-25-F	TAAGGCTAACCTGACTGCACAGATCAACAACTGGCTTAAGTGTAGGCTGGAGCTGCTTCG	
Red-Z0022-25-R	TAAAAGAATATAGACTCAATGTGATTAATAAAAAGCCACTTCACATATGAATATCCTCCTTA	
conf-pKD4-F	CCTTCTTGACGAGTTCTTCT	
Conf-Z0020-R	AGTTAAGGTGGGGGAGATAG	
Conf-Z0021-F	CCTGTGGATTGACCAATGTC	
Conf-Z0021-R	ACGACTTCGGACTTTAAACC	
Conf-Z0022-25-R	GATTAATAAAAAGCCACTTCA	
prom <i>flhD</i> -F	GCGCGGATCCCATTATTTCCACCCAGAATAACC	
prom <i>flhD</i> -R	GCGCCTCGAGGCATCCTGAATTAACCTATCAAG	
prom <i>fliE</i> -F	GCGCGGATCCTATCGCTGACATTTTCATCTCCTG	
prom <i>fliE</i> -R	GCGCCTCGAGTGGGCGTGAATATTACCGTTACC	
prom <i>fliL</i> -F	GCGCCTCGAGGCCAGCATATTTTCGCTGTTCACC	
prom <i>fliL</i> -R	GCGCGGATCCGTAATCAGTCATGTGTTGCGGGTC	
prom <i>flhB</i> -F	GCGCGGATCCCCTCGTCAGACAGCTCGCCAATCC	
prom <i>flhB</i> -R	GCGCCTCGAGAGCCAGTCAGGATCAGGTGGACG	
prom <i>flgB</i> -F	GCGCCTCGAGGGTGGAGGCTGTTGTTTTTGCCGCTC	
prom <i>flgB</i> -R	GCGCGGATCCCTTATCGAGCATATCTCCTCCGCAG	
prom <i>flgA</i> -F	GCGCGGATCCTATTGCCAGATTTTCGCCCCAG	
prom <i>flgA</i> -R	GCGCCTCGAGATTGGCGATGTTTGTGTCGCCAGCAC	
prom <i>fliF</i> -F	GCGCCTCGAGTGGCGAGTGCCTGCGCAGG	
prom <i>fliF</i> -R	GCGCGGATCCAGTCCGATTTCATCGCGCACCTCGTG	
prom <i>fliA</i> -F	GCGCGGATCCGAGTGAATTCACGATAAACAGCCCTG	
prom <i>fliA</i> -R	GCGCCTCGAGGCAACTCCTGCGACAACCACTCCAG	
prom <i>fliC</i> -F	GCGCGGATCCGACTTGTGCCATGATTCTGTTATCC	
prom <i>fliC</i> -R	GGGCCTCGAGGCGATTTTCCTTTATCATTTCGACA	
prom <i>motA</i> -F	GCGCGGATCCTAAGATAAGCAGCAGATCATCCTTC	
prom <i>motA</i> -R	GCGCCTCGAGCTTCGATGTTCTGTAATGCATGG	
qRT- <i>flhD</i> -F	ACCTCCGAGTTGCTGAAACAC	
qRT- <i>flhD</i> -R	TTGCTGGAGATCGTCAACGC	
qRT- <i>flgM</i> -F	CCGTTCAACCGCGGAAACC	
qRT- <i>flgM</i> -R	TGCTGCCGGGTTGCATCAGT	
qRT- <i>motA</i> -F	GCGAACAGTCTGGCGCTGGT	
qRT- <i>motA</i> -R	TGTGCGATAAGCGCCCCAG	
qRT- <i>fliC</i> -F	TGACGGTGCCTCTCTGACATTC	
qRT- <i>fliC</i> -R	AAGACTTCGCAGCATCACTGG	
qRT- <i>icdA</i> -F	ACGTGATTGCTGATGCATTCTGTC	
qRT- <i>icdA</i> -R	ACCGTTCAGGTTTCATACAGGCGAT	
pSU315- <i>flhC</i> -F	CCACAACCTGCTGGATGAACAGAGGTACAGGCTGTTTATCCGTATGATGTTCTCTGAT	
pSU315- <i>flhC</i> -R	GTTACCGCTGCTGGAATGTTGCGCCACACCGTATCAGCATATGAATATCCTCCTTAG	
conf- <i>flhC</i> -F	CAGAGCCAGCAGATCCATATAC	
conf- <i>flhC</i> -R	GTTTGTGTAATGGCGTCGATGC	
Z0021-F	GCGCAAGCTTATGAAGTGGCTTTTATTAATC	
Z0021-R	GCGCGAATTCTTATAAGATGACTTCCTGGAAG	
<i>flhDC</i> -F	GCGCAAGCTTTTAAACAGCCTGTACTCTCTGTTC	
<i>flhDC</i> -R	GCGCGGTACCAATAAGGAGGAAAAAAGTGGGAATAATGCATACCTCCGAG	
<i>araC</i> - <i>flhDC</i> -F	GCGCAGATCTTTATGACAACTTGACGGCTACATC	
<i>araC</i> - <i>flhDC</i> -R	GCGCGTGCACCTGATTTAATCTGTATCAGGCTG	

<sup>a</sup> Bold sequences are restriction sites. The underlined sequence is the optimal ribosome-binding site.

control gene. The primers designed to amplify regions of *flhD*, *flgM*, *motA*, *fliC*, and *icdA* are listed in Table 1. The amplification efficiencies of all primer sets were validated by standard curves. Assays were performed in triplicate with the LightCycler 480 II (Roche) instrument.

**Construction of pFLAG-Z0021 and pFLAG-Z0021-*flhDC*.** Z0021 and *flhDC* were amplified from *E. coli* O157:H7 strain EDL933 chromosomal DNA with primers Z0021-F and Z0021-R and primers *flhDC*-F and *flhDC*-R, respectively. Z0021 was cloned into the HindIII/KpnI sites of

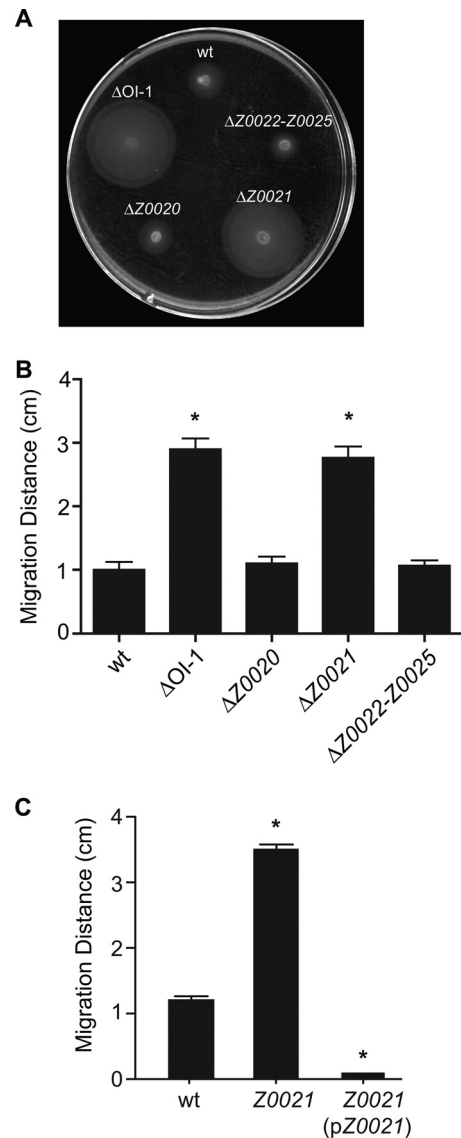
pFLAG-CTC to create pFLAG-*Z0021*, while *flhDC* was cloned into pBAD33 (11) at the KpnI/HindIII sites. A fragment containing *araC-P<sub>BAD</sub>-flhDC* was amplified from pBAD33-*flhDC* with primers *araC-flhDC-F* and *araC-flhDC-R* and cloned into the BglII/SalI sites of pFLAG-*Z0021*. The resulting plasmid, pFLAG-*Z0021-flhDC*, had an IPTG-inducible copy of *Z0021* and a tightly regulated copy of *flhDC* under arabinose control.

**Sequencing of *Z0021* from an STEC strain collection.** The *Z0021* gene was amplified from purified chromosomal DNA by PCR using forward primer 5'-AAG CGG ACG CTA TTA CAA TTA G-3' and reverse primer 5'-GTC CCG ATG GTT CGC CAT TAA C-3' to generate a 721-bp product. DNA was purified through Sephadex and sequenced using a BigDye Terminator sequencing kit (Applied Biosystems, Life Technologies, Carlsbad, CA) and an ABI 3730XL automated sequencer (Applied Biosystems). Sequencing of amplified fragments was performed in both directions and in duplicate. A consensus sequence was generated from four sequence reads per strain using Discovery Studio Gene software (Accelrys Software Inc., San Diego, CA).

## RESULTS

**Deletion of OI-1 increases motility and flagellin production in *E. coli* O157:H7.** Given the existence of flagellar regulatory proteins encoded within fimbrial gene clusters in other bacteria (19, 25, 31), we sought to determine whether the putative fimbrial genomic island OI-1 encodes any repressors of motility. We created a  $\Delta$ OI-1 mutant strain in which the genes *Z0020* to *Z0025* were replaced with a kanamycin resistance cassette and examined the swimming motility of the resulting mutant. In a standard soft-agar motility assay, the  $\Delta$ OI-1 mutant strain was highly motile, in contrast to the wild-type EDL933 strain (Fig. 2A and B). To elucidate which gene(s) was responsible for this phenotype, a series of single- and multiple-deletion mutants was constructed and assayed for motility. The  $\Delta$ *Z0021* mutant strain showed enhanced swimming motility similar to that produced by the complete deletion of OI-1, while the motility of the  $\Delta$ *Z0020* and  $\Delta$ *Z0022-Z0025* mutants was comparable to that of wild-type EDL933 (Fig. 2A and B). All of the strains exhibited growth kinetics similar to those of the wild-type parental strain (data not shown). The enhanced-motility phenotype of the  $\Delta$ *Z0021* mutant strain was eliminated upon complementation with *Z0021* on a plasmid (Fig. 2C). These data indicate that deletion of *Z0021* is responsible for the increased-swimming phenotype of the  $\Delta$ OI-1 mutant strain.

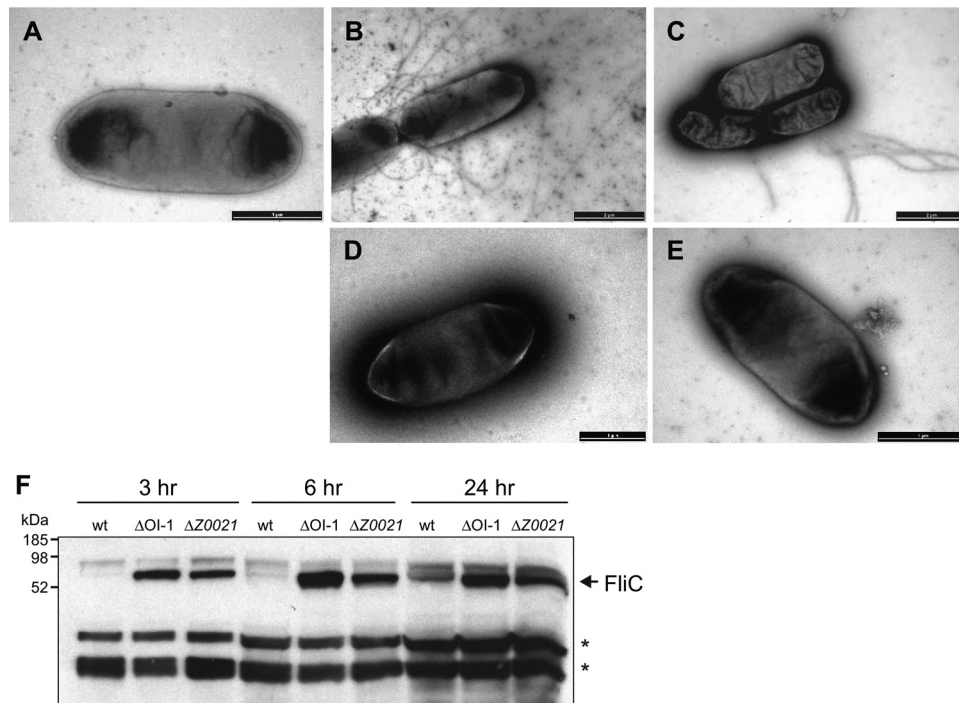
We then investigated whether the enhanced motility of the  $\Delta$ OI-1 and  $\Delta$ *Z0021* mutants was due to an increase in flagellar motility function or to an increase in flagellar biosynthesis. Bacteria were harvested from motility plates and examined by TEM to visualize surface flagella. While the majority of the wild-type EDL933 bacteria were either nonflagellated or possessed a limited number of surface flagella, the  $\Delta$ OI-1 and  $\Delta$ *Z0021* mutants displayed a high number of flagella on their surface (Fig. 3A to C), suggesting that loss of *Z0021* derepresses the biosynthesis of flagella under these conditions. The complemented  $\Delta$ OI-1 and  $\Delta$ *Z0021* mutants possessed a limited number of or no surface flagella, similar to wild-type EDL933 bacteria (Fig. 3D and E). To further confirm that the enhanced motility of the mutant strains was due to an increase in flagellar biosynthesis, the amount of flagellin produced by the *Z0021* and OI-1 deletion mutants was compared to that of the wild type at 3, 6, and 24 h of growth. As shown in Fig. 3F, the  $\Delta$ OI-1 and  $\Delta$ *Z0021* mutants showed higher levels of flagellin production than the wild type at all of the time points examined. Taken together, these data are consistent with



**FIG 2** Deletion of OI-1 increases swimming motility. (A) Wild-type (wt) EDL933 and  $\Delta$ OI-1,  $\Delta$ *Z0020*,  $\Delta$ *Z0021*, and  $\Delta$ *Z0022-Z0025* mutant strains were assessed for swimming motility in a standard soft-agar motility assay. (B) Migration distance in motility agar plates was quantified after 6 h at 37°C. (C) Complementation of the *Z0021* deletion restores the nonmotile phenotype. All data are means and standard deviation from three independent experiments. \*,  $P < 0.001$  (compared to the wild type).

OI-1-encoded *Z0021* as a repressor of motility in *E. coli* O157:H7 strain EDL933.

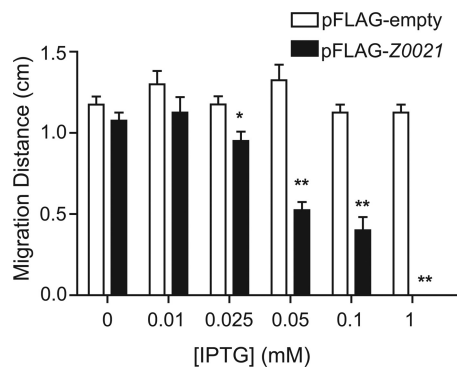
**Overexpression of *Z0021* inhibits motility in *E. coli* O157:H7.** Having identified *Z0021* as a potential negative regulator of flagellum-based motility, we investigated whether elevated expression of *Z0021* would repress the motility of *E. coli* O157:H7 strain EDL933. *Z0021* was cloned under the control of the *tac* promoter into pFLAG-CTC, and the motility of wild-type EDL933 carrying pFLAG-*Z0021* was assessed at various concentrations of IPTG. Increasing inducer concentrations from 0.05 to 1 mM led to a marked decrease in the swimming motility of *E. coli* O157:H7 strain EDL933, which was completely abolished at 1 mM IPTG



**FIG 3** Deletion of *Z0021* increases flagellum production in *E. coli* O157:H7 strain EDL933. (A to E) Transmission electron micrographs of wild-type *E. coli* O157:H7 strain EDL933 (A; scale bar, 1  $\mu$ m), a  $\Delta$ OI-1 mutant strain (B; scale bar, 2  $\mu$ m), a  $\Delta$ Z0021 mutant strain (C; scale bar, 2  $\mu$ m), a  $\Delta$ OI-1(pZ0021) mutant strain (D; scale bar, 1  $\mu$ m), and a  $\Delta$ Z0021(pZ0021) mutant strain (E; scale bar, 1  $\mu$ m). Cells were harvested from motility agar after 6 h at 37°C, stained with 0.1% uranyl acetate, and viewed by TEM. (F) Western blot detection of flagellin (FliC) in the wild-type (wt) and  $\Delta$ OI-1 and  $\Delta$ Z0021 mutant strains. Asterisks indicate nonspecific cross-reacting bands used as a loading control.

(Fig. 4). Western blot analysis confirmed that these motility patterns correlated with elevated levels of *Z0021* (data not shown).

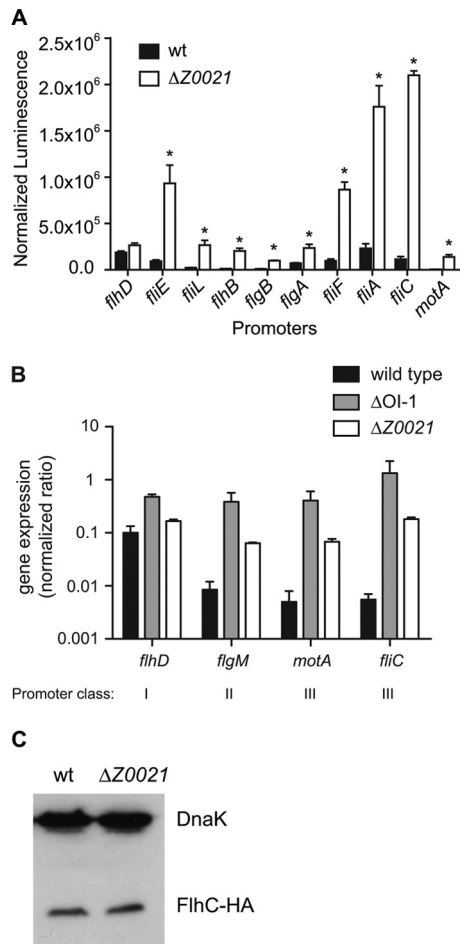
**Deletion of *Z0021* increases the transcription of class II and III promoters.** The enhanced swimming motility of the  $\Delta$ Z0021 mutant was associated with an increase in flagellum production. To examine where in the flagellar activation cascade this point of regulation occurs, we cloned promoter regions corresponding to all three of the promoter classes as transcriptional fusions to *lux-CDABE* and measured the luciferase activities in wild-type EDL933 and  $\Delta$ Z0021 mutant cells. While the *flhDC* promoter ac-



**FIG 4** Overexpression of *Z0021* represses swimming motility in *E. coli* O157:H7 strain EDL933. The motility of *E. coli* O157:H7 harboring an empty pFLAG vector or a pFLAG-*Z0021* vector was assessed at various concentrations of IPTG. The migration distances on motility plates were quantified, and the data represent means and standard deviations. \*,  $P < 0.01$ ; \*\*,  $P < 0.001$  (compared to the empty-vector control).

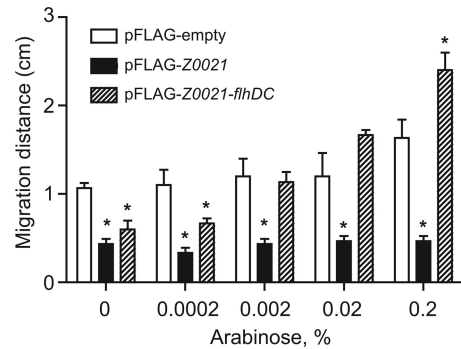
tivity of the  $\Delta$ Z0021 mutant strain was comparable to that of the wild-type strain, there was a significant increase in the activity of the transcriptional reporters for all of the class II and III promoters tested in the  $\Delta$ Z0021 mutant strain compared to that in the wild-type strain (Fig. 5A). These data suggest that *Z0021* acts downstream of the transcription of *flhDC* and prior to the activation of the middle genes. To elucidate whether the increase in class II/III promoter expression was due to increased FlhD and FlhC production, we first used qRT-PCR to measure *flhD*, *flgM*, *motA*, and *fliC* transcript levels in the wild-type and  $\Delta$ Z0021 mutant strains. Deletion of *Z0021* led to a substantial increase in all middle and late flagellar gene transcripts but did not alter the level of the *flhD* transcript (Fig. 5B). We then replaced the chromosomal copy of *flhC* in the *flhDC* operon with a C-terminally HA-tagged variant and determined the levels of FlhC-HA in the wild-type and  $\Delta$ Z0021 mutant backgrounds by Western blot analysis. As expected from the reporter data, the levels of FlhC in the  $\Delta$ Z0021 mutant were comparable to the levels in the wild type (Fig. 5C). These results suggest that *Z0021* regulates flagellar synthesis after the translation of the FlhD and FlhC subunits.

**FlhDC is a high-copy suppressor of the nonmotile phenotype associated with the overexpression of *Z0021*.** Since *Z0021* exerts its regulatory effect on class II flagellar promoters without altering the levels of the master regulator, we considered that *Z0021* was inhibiting the action of a functional FlhD<sub>4</sub>C<sub>2</sub> complex. If this was the case, overexpression of *flhDC* should suppress the nonmotile phenotype associated with elevated levels of *Z0021*. To investigate this, we tested whether increasing expression of *flhDC* would restore motility to *E. coli* O157:H7 expressing *Z0021*. *E. coli*



**FIG 5** Deletion of *Z0021* disrupts flagellar biosynthesis at the level of class II/III activation. (A) Transcriptional reporter activity for class I, II, and III promoters was determined in the wild-type (wt) EDL933 and  $\Delta Z0021$  mutant strains. Luminescence was measured after 6 h of growth at 37°C and normalized to the OD<sub>600</sub>. Data represent means and standard deviations. \*,  $P < 0.001$  (compared to the wild-type control). (B) qRT-PCR analysis of *flhD*, *flgM*, *motA*, and *fliC* in the wild-type and  $\Delta Z0021$  mutant strains. All assays were performed independently with three technical replicates, and the levels of all transcripts were normalized to that of *icdA* mRNA. The data represent the means and standard deviations of two independent experiments. (C) Wild-type EDL933 and  $\Delta Z0021$  mutant whole-cell lysates were collected at an OD<sub>600</sub> of ~0.5, and the levels of FlhC-HA were determined by Western blotting. DnaK served as a loading control.

O157:H7 carrying pFLAG-*Z0021*-*flhDC* has a tightly regulated copy of *flhDC* under arabinose control and an IPTG-inducible copy of *Z0021*. The motility of this strain was assayed at various concentrations of arabinose and at a fixed concentration of 0.1 mM IPTG—a concentration which we showed impairs the motility of *E. coli* O157:H7 strain EDL933 harboring pFLAG-*Z0021* (Fig. 4). In our soft-agar motility assay, an arabinose concentration of 0.002% restored the motility of *E. coli* O157:H7 strain EDL933 harboring pFLAG-*Z0021*-*flhDC* to that of the empty-vector control, while arabinose concentrations of  $\geq 0.02\%$  increased the motility of *E. coli* O157:H7 strain EDL933 harboring pFLAG-*Z0021*-*flhDC* beyond that of the empty-vector control (Fig. 6). Taken together, these data show that the motility defects caused by *Z0021* can be suppressed by increasing *flhDC* expression, imply-



**FIG 6** Increased expression of *flhDC* suppresses the nonmotile phenotype associated with *Z0021*. The motility of *E. coli* O157:H7 strain EDL933 harboring an empty pFLAG vector, pFLAG-*Z0021*-*araC*-P<sub>BAD</sub>, or pFLAG-*Z0021*-*flhDC* in 0.25% LB agar containing ampicillin and 0.1 mM IPTG was assessed. Arabinose was varied from 0 to 0.2%, and migration distance was measured after 6 h of growth at 37°C. Data represent means and standard deviations. \*,  $P < 0.01$  (compared to the pFLAG-empty vector).

ing a genetic link between *Z0021* and the FlhD<sub>4</sub>C<sub>2</sub> regulatory complex.

**Prevalence of *Z0021* among STEC strains.** OI-1 was previously identified in seropathotype A strains of STEC. To examine the distribution of *Z0021* among the other four seropathotypes of non-O157:H7 STEC, we screened the 69 strains from the original seropathotype collection of STEC strains (15) by PCR and sequenced the positive *Z0021* gene products. Only the O157:H7 strains in the seropathotype A group had the same *Z0021* sequence as the EDL933 reference strain with 100% conservation (Fig. 7). Although strains of the other seropathotypes harbored the *Z0021* gene, the gene contained either a frameshift mutation at nucleotide position 148 resulting in a downstream premature stop codon at position 73 or a deletion mutation at position 299 resulting in a frameshift starting at amino acid position 100 (Fig. 7). Interestingly, these two gene variants appear to have evolved a number of times independently in different serotypes that assort to different seropathotype classes. Only seven other strains in the collection had a *Z0021* sequence similar to that of the EDL933 reference strain; however, in all of the cases, these sequences had at least one nonsynonymous substitution. Thus, only the O157:H7 strains in our collection had a *Z0021* sequence that was identical to that of the EHEC reference strain. This *Z0021* sequence from all seropathotype A strains was distinguished from those of all other serotypes by unique frameshifts or nonsynonymous substitutions.

## DISCUSSION

Many regulators of the flagellar transcriptional hierarchy have been identified, and some of these are encoded within fimbrial gene clusters, presumably to control the opposing processes of adherence and motility. In the *E. coli* O157:H7 genome, the majority of the putative fimbrial gene clusters are uncharacterized and no fimbrial-operon-encoded regulators of motility have been identified to date. In this paper, we have reported on a novel repressor of *E. coli* O157:H7 motility encoded in the putative type 1-like fimbrial gene cluster OI-1 and have shown that *Z0021* regulates flagellar synthesis through its influence on class II promoters via FlhD<sub>4</sub>C<sub>2</sub>.

*E. coli* type 1 fimbriae have an established role in mediating adherence to host cells, and the closely related type 1-like P fim-

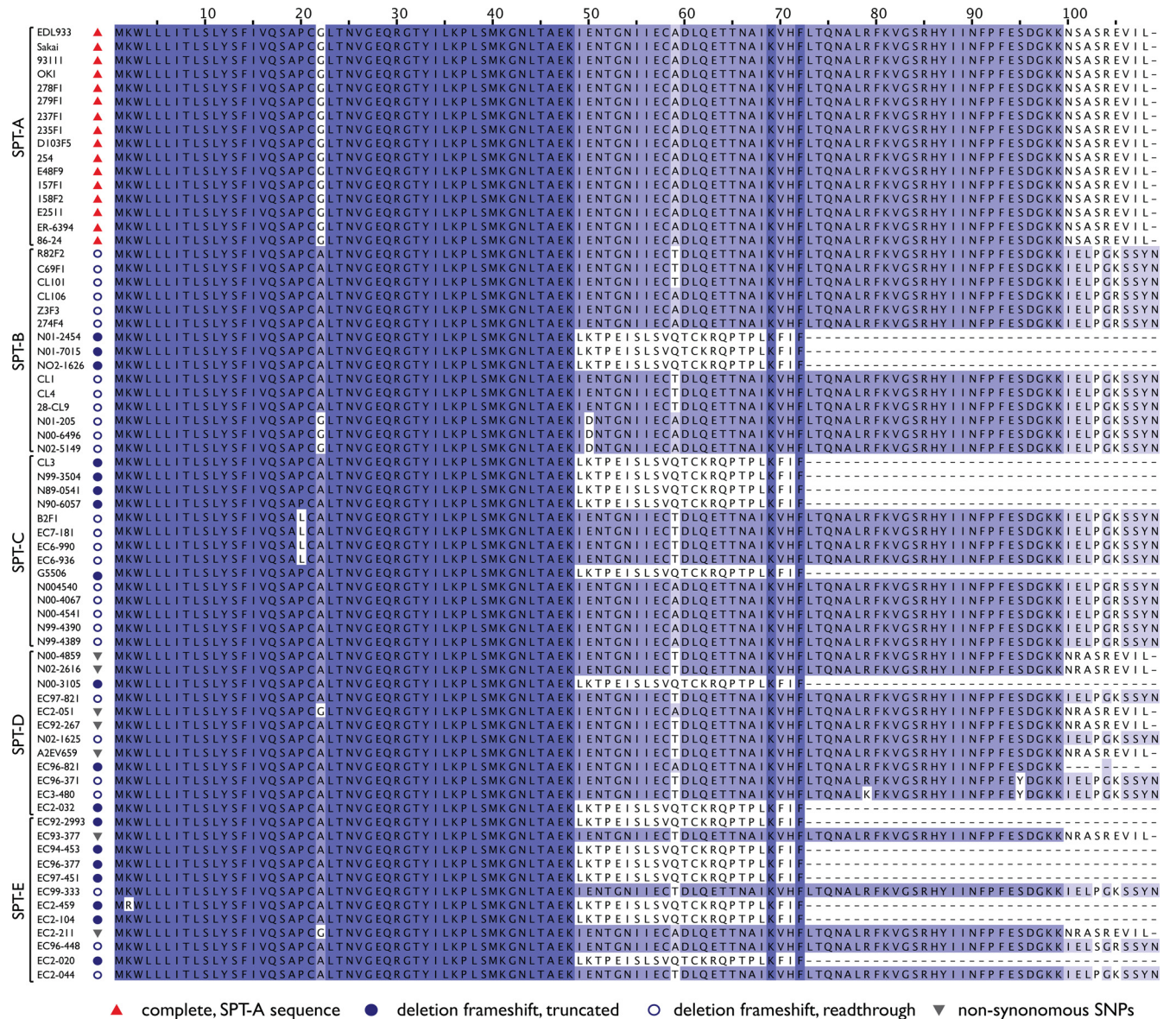


FIG 7 Prevalence of *Z0021* in STEC strains. *Z0021* genes were amplified from 69 STEC strains, including non-O157 STEC strains of all seropathotypes. Nucleotide sequences were translated *in silico*, and the amino acid sequences were aligned. Strain names are given to the left of the sequences and grouped into seropathotypes (SPTs). The alignment is shown as a heat map according to the conservation at each position, with dark blue representing higher conservation and light blue representing lower conservation among all of the sequences. SNPs, single-nucleotide polymorphisms.

briae have been shown to be important for the pathogenesis of uropathogenic *E. coli* (6, 23, 29). Since *E. coli* O157:H7 strains are unable to produce type 1 fimbriae because of a deletion in the *fim* regulatory region (28), novel type 1 or type 1-like fimbriae may play a role in the process of intestinal colonization. OI-1 is one of four fimbria-encoding OIs that are found in seropathotype A strains of O157 STEC and may be a key determinant of the unique pathogenesis of such strains (30). OI-1 may encode type 1-like fimbriae on the basis of its similarity to the *E. coli* type 1 fimbrial locus. In comparison to the LPF clusters (OI-141 and OI-154) which contain mutations in their export machinery (20), *Z0022* and *Z0023* of OI-1 encode potentially functional usher and chaperone proteins, respectively. OI-1 could therefore produce fimbriae without complementation from another fimbrial gene clus-

ter. The putative adhesin gene in OI-1, *Z0020*, is not similar to characterized adhesin genes, suggesting that it may encode a novel adhesin or that other components of the fimbriae produced by OI-1 confer binding specificity (20). Future studies are required to structurally and functionally characterize the fimbriae produced by OI-1 and to determine the importance of this OI in O157 STEC pathogenesis.

In an analysis of the expression of 16 putative fimbrial gene clusters in *E. coli* O157:H7, OI-1 showed no or minimal expression under the *in vitro* conditions tested (20). While the expression of OI-1 *in vivo* has not been examined, our work has shown that the expression of OI-1, and in particular *Z0021*, is important for the repression of flagellar synthesis in *E. coli* O157:H7. The expression of flagella has been shown to vary during the course of



an infection; flagella are produced extensively by attaching bacterial cells in the early stages of infection but are not present in bacterial microcolonies that form at later time points (22). We therefore speculate that the gene products of OI-1 may be of significance following the early stages of infection at a time when flagellar expression is diminished.

Z0021 was found to exert its regulatory effects on class II flagellar promoters downstream of *flhDC* transcription and translation. It is therefore conceivable that Z0021 could regulate flagellar synthesis by (i) targeting FlhD and/or FlhC for degradation or (ii) preventing a functional FlhD<sub>4</sub>C<sub>2</sub> complex from binding to class II promoters. The ClpXP protease has been shown to degrade FlhD<sub>4</sub>C<sub>2</sub> in both *S. enterica* serovar Typhimurium and *E. coli* O157:H7 (18, 34). Given that the transcript and protein levels of FlhD and FlhC were unchanged in the wild-type and ΔZ0021 mutant strains, this suggests that Z0021 regulates flagellar synthesis through a mechanism independent of ClpXP. The mechanism of action of Z0021 may be more similar to that of *S. enterica* serovar Typhimurium YdiV, which, in addition to repressing flagellar synthesis via a ClpXP-dependent pathway (33), also acts via a ClpXP-independent pathway by interacting with the FlhD<sub>4</sub>C<sub>2</sub> complex and preventing its binding to class II promoters (38). However, we have been unable to identify motifs shared by Z0021 and YdiV and the two proteins lack sequence conservation at the amino acid level. The latter proposed mechanism for Z0021-mediated repression is supported by the ability of *flhDC* to suppress the nonmotile phenotype associated with the expression of Z0021, indicating that the effects of Z0021 are reversible and dependent on the levels of the FlhD and/or FlhC subunits.

Comparative sequence analysis of the Z0021 gene in STEC strains from our collection revealed a high degree of nucleotide sequence conservation among seropathotype A strains, including those which are nonmotile. FlhD<sub>4</sub>C<sub>2</sub> not only acts as the master regulator of the flagellar cascade but has also been shown to bind to and activate nonflagellar genes (32). Z0021 may therefore be conserved among these nonmotile strains to exert a regulatory effect on nonflagellar genes via an interaction with FlhD<sub>4</sub>C<sub>2</sub> or the FlhD and FlhC subunits. A highly conserved Z0021 gene is also present in some seropathotype B to E strains. Although OI-1 is found predominantly in seropathotype A strains, there is evidence of OI-1 in other seropathotypes, which may account for the wider distribution of Z0021 in non-O157 STEC strains (30).

In summary, we identified a novel repressor of motility in *E. coli* O157:H7 and showed that Z0021 negatively regulates flagellar synthesis at a stage prior to the transcription of the middle and late genes. The characterization of Z0021 is the first report of a fimbrial-operon-encoded gene product that represses flagellar synthesis in *E. coli* O157:H7, and consistent with this role, we propose that Z0021 be renamed *fmrA* (fimbrial-operon-encoded motility regulator A). Most importantly, our work has provided insight into the function of an OI that may be associated with the enhanced pathogenesis of seropathotype A strains of STEC.

## ACKNOWLEDGMENTS

This work was funded by an operating grant to B.K.C. from the Canadian Institutes of Health Research (MOP-82704), an infrastructure grant from the Canada Foundation for Innovation, and the Canada Research Chairs Program from the Government of Canada. S.E.A. is the recipient of a Canada Graduate Scholarship from the CIHR. U.S. was supported by an

NSERC Visiting Fellowship. B.K.C. is the Canada Research Chair in Infectious Disease Pathogenesis.

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