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Identification of Enterohemorrhagic *Escherichia coli* O26:H$^-$ Genes Required for Intestinal Colonization in Calves

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Enterohemorrhagic *Escherichia coli* (EHEC) infections in humans are an important public health problem and are commonly acquired via contact with ruminant feces. The serogroups that are predominantly associated with human infection in the United States and Europe are O157 and O26. Serotypes O157:H7 and O26:H$^-$ differ in their virulence and tissue tropism in calves and therefore may colonize calves by distinct mechanisms. The mechanisms underlying EHEC intestinal colonization and pathogenesis are poorly understood. Signature-tagged mutagenesis was used to identify 59 genes of EHEC O26:H$^-$ that are required for the intestinal colonization of calves. Our results indicate important roles for locus of enterocyte effacement (LEE)-encoded type III secreted proteins in intestinal colonization. In addition, colonization is facilitated by cytotoxins, putative type III secreted proteins unlinked to the LEE, a putative fimbrial operon, and numerous genes involved in central metabolism and transport and genes of unknown function. Our data also imply that the elaboration of type I fimbriae by EHEC O26:H$^-$ is disadvantageous for persistence within the bovine intestines. These observations have important implications for the design of vaccines to control these important zoonotic pathogens.

Enterohemorrhagic *Escherichia coli* (EHEC) comprises a group of zoonotic enteric pathogens which characteristically produce one or more Shiga toxins and form attaching and effacing (AE) lesions on intestinal epithelia (43). In humans, EHEC infections can result in bloody or nonbloody diarrhea, which may be complicated by hemorrhagic colitis and severe renal and neurological sequelae, including hemolytic-uremic syndrome (47).

The EHEC serogroup predominantly associated with human infections in the United States and Europe is O157, but other serogroups like O26, O103, and O111 are also frequently isolated (4, 22). Ruminants are an important reservoir of EHEC (21), and human infections are frequently associated with contact with ruminant feces (31). Clinical signs of natural EHEC infections in calves may vary from subclinical to dysentery depending on the serogroup. Natural and experimental infection of normal cattle with *E. coli* O157:H7 results in efficient colonization of the intestinal tract in the absence of clinical signs (7, 61). In contrast, EHEC serogroups O5, O26, and O118 are commonly associated with diarrhea in farm animals, which imposes a significant economic burden on livestock producers (9, 48, 60). Extensive adherence and AE-lesion formation occur with O5, O26, and O111 in the bovine large intestine (48, 58), whereas EHEC O157 has been reported to exhibit tropism for lymphoid follicle-dense mucosa in the terminal rectum (44). Together, these observations suggest that EHEC O157:H7 and non-O157:H7 may colonize the bovine intestines by distinct mechanisms. Strategies to lower the prevalence of EHEC in cattle offer the possibility of reducing the incidence of human infections. An understanding of the mechanisms by which EHEC colonizes the ruminant intestines is necessary for the development of effective intervention strategies.

AE-lesion formation, in which bacteria adhere intimately to the apical surfaces of enterocytes on raised actin-rich pedestals, is determined by the chromosomal locus of enterocyte effacement (LEE), which encodes a type III protein secretion system (20). In EHEC, several proteins are exported by this secretion system, some of which subvert pathways that regulate the host cell cytoskeleton and bring about pedestal formation (20). The LEE-encoded secreted protein Tir is translocated into the host cell membrane, where it acts as a receptor for the bacterial outer membrane protein intimin (14). Intimin is a key colonization factor for EHEC in neonatal calves (12), adult cattle, and sheep (10). However, intimin-null mutants still colonize some sites of the ruminant gastrointestinal tract, indicating that other colonization factors may also be important.

Some of the factors mediating colonization by EHEC have recently been reviewed (57). EHEC factor for adherence (*efa-1*) influences colonization of the bovine intestine by non-O157 EHEC. Mutation of *efa-1* in EHEC serotypes O5 and O111 significantly reduced fecal shedding and bacterial adherence to the colonic epithelium in experimentally infected calves (58). All non-O157 EHEC strains tested and related enteropathogens such as *Citrobacter rodentium*, *Hafnia alvei*, and rabbit enteropathogenic *E. coli* (EPEC) have the *efa-1* gene (28, 45). In contrast, *E. coli* O157:H7 strains lack a full copy of *efa-1* (23, 49), reinforcing the notion that O157:H7 and non-O157:H7 EHEC may colonize the intestines by distinct mechanisms. Several other factors, like Shiga toxins, flagella, fimbriae, and other surface appendages, have been proposed as candidate colonization factors, although their role is unclear.

In this study, we report the identification of novel EHEC O26:H$^-$ genes that are required for intestinal colonization in calves by using signature-tagged transposon mutagenesis (STM). This analysis has revealed new targets for the control

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and diagnosis of EHEC infections and provided insights into EHEC pathogenesis.

MATERIALS AND METHODS

Bacterial strains and media. EHEC O26:H$^-$ strain 193 is an stx_1-positive *eae*β *E. coli* which was originally isolated in 1962 from a calf with diarrhea in the United States (32). EHEC O157:H7 strain EDL933 was first reported in 1982 following an outbreak of hemorrhagic colitis in the United States (50). Strains EDL933nal^R and -193nal^R are spontaneous nalidixic acid-resistant derivatives of EDL933 and -193, respectively, and exhibit normal growth and adhesion characteristics in vitro. NADC5738 is a nalidixic acid-resistant derivative of nonpathogenic porcine commensal *E. coli* strain 123 (O43:H28) (11). The 95 mini-Tn*5*Km2-tagged transposons were donated by C. M. Tang and maintained in *E. coli* S17.1λ*pir* for conjugation (25). Bacteria were isolated on Luria-Bertani (LB) or sorbitol MacConkey-tellurite (2.5 μg/ml) agar (T-SMC) and cultivated in LB broth with appropriate antibiotics: ampicillin (Amp) at 100 μg/ml, nalidixic acid (Nal) at 25 μg/ml, and/or kanamycin (Km) at 50 μg/ml.

Bovine oral challenge model. All animal experiments were performed in accordance with the Animals (Scientific Procedures) Act of 1986 and were approved by the local Ethical Review Committee. An oral inoculation model in calves was used as described previously (58) with a minor modification. For this study, a total of 31 4-day-old conventional calves were orally challenged with ~10^{10} CFU of EHEC without antacid just before morning feeding. Prior to challenge, calves were confirmed to be culture deficient for EHEC and *Salmonella* spp. by direct plating of rectal swabs on T-SMC or brilliant green agar, respectively. While it is acknowledged that T-SMC agar may not detect all EHEC cells, it is highly selective for serogroups O157 and O26, which are by far the most prevalent in cattle in the United Kingdom at present. Presumptive EHEC colonies were screened for stx_1 and stx_2 genes by PCR using the primer pairs Stx1F (5′-ATAAATCGCCATTCGTTGACTAC-3′) with Stx1R (5′-AGAACGCCCA CTGAGATCATC-3′) and Stx2F (5′-GGCACTGTCTGAAACTGCTCC-3′) with Stx2R (5′-TCGCCAGTTATCTGACATTCTG-3′). Calves excreting *stx*-positive *E. coli*, excreting salmonellae, or in general having poor health status were excluded from the study. Also, prechallenge fecal samples were confirmed to be culture deficient on T-SMC agar with the appropriate antibiotics. All calves received colostrum from their respective dams for the first 24 to 48 h before being transported to the experimental high-security unit.

Viable EHEC per gram of feces was enumerated twice daily by plating triplicate 10-fold serial dilutions onto T-SMC–Nal plates for wild types or T-SMC–Nal-Km plates for mutants. Where appropriate, the fecal shedding data were statistically analyzed after a 10 log transformation for the effect of mutation by means of an F test, with the data taken as repeated measurements (Proc Mixed Statistical Analysis System; SAS Institute, Cary, N.C.).

Tissue sampling, sectioning, and confocal microscopy were carried out as described previously (58). As a rule, the middle loop of the spiral colon was sampled (centripetal turn). A complete circumferential section was excised (approximately 10 cm), and full-thickness biopsies were taken in triplicate. Triplicate sections were examined.

Construction and screening of the STM mutant bank. The mutant bank was constructed largely as described by Hensel et al. (25), with minor modifications. The signature-tagged transposons were transferred to EHEC O26:H$^-$ strain 193nal^R in 95 individual conjugative matings. Insertion mutants were selected on T-SMC–Nal-Km plates, and 2,850 mutants were arrayed into 30 96-well microtiter plates such that each mutant could be distinguished from the others by a unique sequence tag. Colonies were tested for sensitivity to Amp to avoid mutants with episomal replication of pUTmini-Tn*5*Km2 or integration of the plasmid into the chromosome.

Overnight LB cultures of 95 uniquely tagged mutants (450 μl each) were pooled. Approximately 10^{10} CFU of bacteria (20 ml) were orally fed to a single calf just before the morning feed. One milliliter was pelleted and frozen to represent the input pool of tags. In total, 570 mutants (six pools of 95 mutants each) were screened, one pool per calf (six calves total). Output pools from feces and colonic mucosa were collected 5 days postinfection. For each output pool, approximately 10,000 colonies were pooled and used for the preparation of genomic DNA for generation of the hybridization probe (25). Signature tags from input and output pools were amplified and labeled from genomic DNA by PCR as described previously (25).

Mapping of transposon insertion sites and DNA sequence analysis. The transposon junctions from each attenuated mutant were cloned by ligation of EcoRI- or EagI-restricted genomic DNA into similarly restricted pBluescript KS(+) and transformed into chemically competent *E. coli* TOP10F′ cells (Invitrogen Ltd., Paisley, United Kingdom). Transformants were selected on LB-Amp-Km plates. Plasmid DNA was isolated using a Qiaprep spin miniprep kit (QIAGEN GmbH, Hilden, Germany), and the insert DNA sequence was obtained using a mini-Tn*5*Km2-specific primer, P6 (5′-CCTAGGCGGCCAGATCTG-3′) for EagI clones and P10 (5′-TCCTCTAGAGTCGACCTGC-3′) for EcoRI clones (Lark Technologies, Inc., Saffron Walden, United Kingdom). Sequences were analyzed using the BLASTN search algorithm.

FAS assay. Fluorescent actin staining (FAS) for the detection of F-actin under sites of bacterial adhesion to HeLa cells was performed as described previously (29).

Detection of EspD by Western blotting. Bacteria were grown to mid-logarithmic phase (A_{600} ≈ 0.8) in minimal essential medium buffered with 25 mM HEPES. Secreted proteins were precipitated with trichloroacetic acid and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by direct staining (GelCode Blue; Perbio Science UK Ltd., Cheshire, United Kingdom). Western blotting was performed as described previously using a monoclonal antibody specific for EspD (39).

***fim* switch orientation and agglutination assay.** Analysis of the orientation of the *fim* switch was carried out as described by Roe et al. (51) with one modification. The products were separated using a 1.5% agarose gel run at 100 V for 30 min. The orientation of the switch was determined in the 193nal^R wild type, the *fimE* mutant, and genomic DNA derived from the input and output pools collected from feces and colonic mucosa after overnight growth on T-SMC agar under identical conditions. At challenge and on day 5 postinoculation, the orientation of the *fim* switch in the inoculum and in bacteria recovered from the feces of infected calves was determined by colony PCR. Yeast agglutination and mannose inhibition of agglutination were performed as described previously (51).

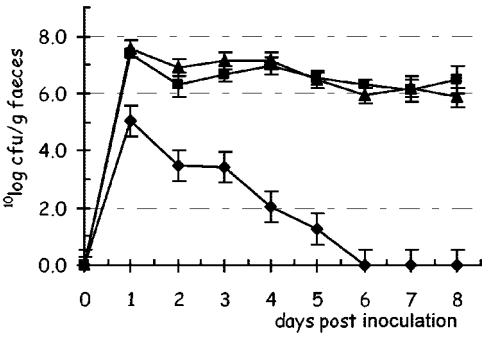

FIG. 1. Least-square means of fecal shedding (± standard errors of the means) of O26:H$^-$ strain 193nal^R (■) in four calves, O157:H7 strain EDL933nal^R (▲) in four calves, and nonpathogenic *E. coli* NADC5738 (◆) in two calves following oral challenge.

RESULTS

It is possible to distinguish colonizing strains from noncolonizing strains by recovering the bacteria from the feces 5 days after inoculation. Experimental infection of four 4-day-old calves with the calf dysentery strain 193nal^R induced transient diarrhea. The bacteria were persistently excreted in the feces for more than 8 days (Fig. 1). Experimental infection of calves (n = 4) with EDL933nal^R failed to induce any clinical signs, yet bacteria were excreted in comparable numbers as strain 193nal^R (Fig. 1). The nonpathogenic *E. coli* strain (NADC5738, n = 2) was excreted in significantly lower numbers ($P < 0.0001$) and was not detectable in the feces by day 6 postinoculation (Fig. 1).

O157:H7 and O26:H$^-$ EHEC efficiently colonize calves by potentially distinct mechanisms. To compare the levels of interaction of EHEC O26:H$^-$ and O157:H7 with intestinal mucosa, immunohistological analysis was carried out on intestinal tissues taken 4 days postinoculation. At that time point, calves were shedding comparable numbers of bacteria (Fig. 1).

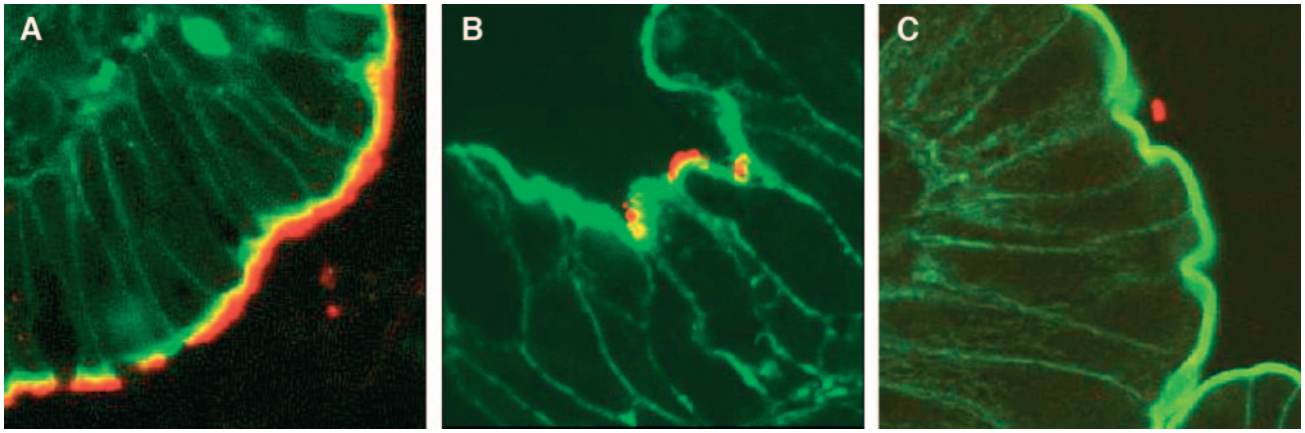

FIG. 2. Confocal laser scanning micrographs showing interactions between EHEC and bovine spiral colonic epithelium. O26:H$^-$ strain 193nal^R (A), O157:H7 strain EDL933nal^R (B), and the 193nal^R *escN*::*Tn* mutant (C) are shown. Bacteria were detected with rabbit antibody specific for the lipopolysaccharide and anti-rabbit immunoglobulin-Alexa^{568} (red), and F-actin in the mucosa was stained with fluorescein isothiocyanate-phalloidin (green).

Strain 193nal^R was seen to be adhering in large numbers to the colonic (Fig. 2A), cecal, and rectal mucosa. In contrast, EDL933nal^R was only rarely seen to be interacting with colonic (Fig. 2B) or other large intestinal mucosa.

Six pools of 95 transposon mutants were screened by oral inoculation of conventional calves. The inoculum dose (pool of 95 mutants) varied between 9.62 and 9.91 10 log CFU per calf. Recoveries higher than 10^5 CFU per gram were routinely obtained in the fecal and mucosal output pools 5 days postchallenge. Eighty-four mutants were absent or poorly represented in the output pools compared with those in the input pool, indicating that the mutants have defects in factors influencing intestinal colonization and/or survival. Sixty-six percent of the mutants absent from the colonic epithelium were also absent in the feces. A total of 19 mutants were completely absent in both output pools, and 16 mutants were absent in one pool and poorly represented or present in the other pool.

Identification of EHEC O26:H$^-$ genes required for intestinal colonization of calves. Table 1 gives an overview of 62 noncolonizing O26:H$^-$ mutants identified by STM, the intensity of the hybridization signal from the output pools recovered from feces and mucosa compared with that from the input pool, the site of transposon insertion relative to the predicted start codon, a brief description of the function of the disrupted gene, and the O157:H7 strain EDL933 homologue, where present. The roles of the major groups of genes are discussed below.

Type III secretion and associated genes. Seven mutants were identified with insertions in genes of the LEE (Table 1). It is most unlikely that the phenotypes of multiple distinct insertions in the LEE are the result of second-site effects.

The genes *escC* and *escN* encode putative structural components of the LEE-encoded type III protein secretion system and are required for EspD secretion, adhesion, and actin nucleation under adherent bacteria as determined by FAS. The gene *espA* encodes a type III secreted protein that is believed to form a channel through which effectors are injected into host cells. Consistent with this, the mutation resulted in reduced secretion of EspD, reduced adherence, and loss of FAS reactivity. Furthermore, a mutant was identified with an insertion in *espD*. EspD is required to form membrane pores and for the formation of EspA filaments (30).

Sequencing of the EHEC O26:H11 LEE (Benkel and Chakraborty ECO277443) identified the gene *st22* upstream of *escV* and *escN*. It encodes a protein of previously unknown function. Mutation of *st22* also resulted in reduced secretion of EspD, reduced adherence, and loss of FAS reactivity, implicating St22 in type III secretion.

In a separate calf oral challenge study using pure cultures of the *escN* mutant or the parent strain, the *escN* mutant was excreted in considerably lower numbers than the parent strain (Fig. 3). On day 4 postchallenge, a 99% reduction in shedding was observed (4.41 versus 6.49 10 log CFU/g of feces). Calves were killed at this point for immunohistological analysis, which showed greatly reduced adherence of the *escN* mutant to the colonic mucosa (Fig. 2C) compared with the parent strain (Fig. 2A).

As with other members of the family *Enterobacteriaceae*, it is likely that other type III secreted effector proteins are encoded within the genome at loci that are unlinked to the LEE. STM analysis has identified two such putative secreted effector proteins disrupted in mutants 3B5 and 3H5 (Table 1). Western blotting indicated that secretion of EspD by mutant 3B5 was normal such that the mutation was not directly influencing the function of the LEE-encoded type III secretion system. Sequencing of the transposon junctions in mutant 3B5 generated a sequence with 87% nucleotide identity to open reading frame (ORF) *z1829* of *E. coli* O157:H7 strain EDL933. Insertion sequences and prophage genes flanking *z1829* suggest that Z1829 has been acquired by horizontal gene transfer. A BLASTP search with Z1829 revealed 31% identity in a 439-amino-acid overlap with GogB of *Salmonella enterica* serovar Typhimurium LT2, a leucine-rich repeat protein encoded within the gifsy-1 prophage. However, the region of homology did not lie within the leucine-rich repeat domains, which have been associated with targeting type III secreted effector proteins to the needle complex (40).

Mutant 3H5 was also defective for intestinal colonization

TABLE 1. EHEC O26:H$^-$ genes required for intestinal colonization in calves[a]

Identities of EHEC O26:H$^-$ genes				Output[b]		EspD[c] secretion	FAS lesions	EDL933 homologue[d]	Insertion point/ gene size (bp)
Mutant	Gene	Function	Description	F	M				
Type III secretion and associated genes									
3G9	*espD*	LEE	Secreted protein	−−	−−	−	ND	*z5106*	771/1,143
4D6	*espA*		Filament	−−	−−	−	−	*z5107*	68/579
4E9	*escN*		Structural protein	−−	−−	±	−	*z5119*	841/1,341
6A8	*st22*		Protein, function unknown	−−	−−	±	−	*z5121*	64/354
ID3	*escC*		Structural protein	−−	−−	−	−	*z5126*	1,173/1,539
6A11	*escC*		Structural protein	−−	−	−	ND	*z5126*	876/1,539
3B5		Unlinked to LEE	Putative secreted effector protein, *S. enterica* serovar Typhimurium GogB orthology	−−	−	+	+	*z1829*	1,095/1,362
3H5	*pkgA*		Putative secreted effector protein	−−	−	+	+	*z4200*	863/1,029
Cytotoxins									
3A7	*ehxA*		Enterohemolysin A (*hlyA*)	+	−	+	+	*l7048*	667/2,997
3E2	*pssA*		Secreted serine protease	−−	−−	+	+	*l7020*	1,362/3,903
3E4	*pssA*		Secreted serine protease	−	−−	+	+	*l7020*	1,601/3,903
4B5	IG[e]		360 bp 5′ of LEE encoded EHEC factor for adherence (*efa-1*)	−−	−	+	+		/9,672
Other surface structures									
2G11	*fimE*		Recombinase, regulator for *fimA*	−−	−−	+	+	*z5911*	427/597
4E3	*ydeR*		Putative fimbrial-like protein, FimG protein homologue	−	−	+	+	*z2205*	485/504
2A12	*rmlB*		dTDP-glucose 4,6-dehydratase; O26 O-antigen cluster	−−	−−	+	+		423/1,086
2C2	*fnl2*		Fnl2 protein; O26 O-antigen cluster	−−	−−	+	+		1,005/1,125
3A9	*wbuA*		Putative rhamnosyltransferase; O26 O-antigen cluster	−−	−−	+	+		559/795
6H3	*traY*		Membrane protein; pO113:H21	−	−	+	ND		1,980/2,166
6E2			Putative membrane protein	±	−	+	+	*z1193/z1633*	174/471
Transport systems									
1B9	*ycjV*		Putative ATP binding component, MalK homologue	+	−	++	ND	*z2463*	558/1,131
1B10	*yddA*		Putative ATP binding component	−−	−	++	ND	*z2212*	633/1,686
2B1	*pitB*		Partial putative transport protein	+	−−	++	ND	*z4341*	230/1,317
4C10	*trkA*		Small molecules: cations, transport of potassium	−	−	+	ND	*z4660*	1,022/1,377
4E8	*mglA*		Small molecules: carbohydrates, organic acids, alcohol	−	−	+	ND	*z3404*	323/1,521
6B6	*yicK*		Two-module transport protein K-12 homologue	+	−	ND	ND	*b3659*	421/1,185
Putative-function genes									
2B2	*evgS*		Putative sensor for regulator *evgA*	−	−	+	+	*z3632*	364/3,594
3A6	*yfdV*		Putative receptor protein, near *evgS*	+	−	+	+	*z3635*	209/945
2B3			Putative tail component of prophage	−	−	+	+	*z2142*	539/699
6C8			Putative oxidoreductase, major subunit	+	−	+	+	*z2207*	857/2,280
6F12	*xis*		Putative excisionase; *S. enterica* serovar Typhi homologue	−	−	+	ND		234/387
6H6			Putative protease encoded within prophage X	−	−−	+	ND[f]	*z1930*	528/1,485
6B3	IG		81 bp upstream of putative helicase	−	−	+	ND[f]	5′ *z1129*	/2,385
Central intermediary metabolism									
1C2	*nrdD*	2′-Deoxyribonucleotide metabolism	Anaerobic ribonucleoside-triphosphate reductase	−	−	+	+	*z5848*	769/2,139
1H12	*trxB*		Thioredoxin reductase	−	−−	+	+	*z1232*	55/966
2A5	*narG*	Energy metabolism, carbon	Anaerobic respiration, nitrate reductase 1, alpha subunit	−	−	+	+	*z2001*	697/3,744
3C5	*tdcE*		Anaerobic respiration, probable formate acetyltransferase 3	+	−−	+	+	*z4466*	2,242/2,295
4B11	IG		177 bp upstream of *acnA*: trichloroacetic acid cycle, aconitate hydrase 1	−	−−	+	ND	5′ *z2532*	/2,676
4B1	*speA*	Polyamine biosynthesis	Biosynthetic arginine decarboxylase	−	−	+	+	*z4283*	1,557/1,977
4C9			Unknown function, between *speA* and *speB*	−	±	+	+	*z4282*	58/732
1G11	*trpB*	Amino acid biosynthesis	Structural, tryptophan synthetase beta subunit	−−	−−	+	+	*z2550*	686/1,194

Continued on facing page

TABLE 1—*Continued*

Identities of EHEC O26:H⁻ genes				Output[b]		EspD[c] secretion	FAS lesions	EDL933 homologue[d]	Insertion point/ gene size (bp)
Mutant	Gene	Function	Description	F	M				
1D11	IG		46 bp upstream of *serA*: serine	−−	−−	++	+	*5′ z4251*	/1,233
2F3	*sanA*		Vancomycin sensitivity	−−	−−	+	+	*z3399*	494/720
2G4	*recD*		Degradation of DNA	−−	−−	+	+	*z4136*	935/1,827
Genes of unknown function									
2B12	*stbA*		Possible stability locus; pC15-1 a	−−	−−	+	ND	Orf27	624/963
2A2	*rhsA*		Repeat regions, rhs element associated	−	−	+	+	*z5014*	3,654/4,134
1B7	*ymgA*		Hypothetical protein; K-12 homologue	−	−	+	ND	*b1165*	205/273
3B4	IG		188 bp downstream of *entD*; K-12 homologue	−−	±	ND	+	*3′ b0583*	/771
1C7	IG		79 bp upstream of *yafA*, pCol1b; *Shigella* homologue	−	−	+	+	*5′ yafA*	/603
2F5			Unknown, phage and prophage related	+	−	+	ND	*z2056*	821/1,050
1A11			Hypothetical protein	+	−	++	ND	*z2749*	1,414/1,899
6D12			Hypothetical protein	+	−	++	+	*z2749*	138/1,899
6A2	*ydiY*		Hypothetical protein YdiY precursor	±	−	+	ND	*z2751*	737/759
4E12	IG		5 bp downstream, unknown	−	−	+	+	*3′ z1182/ z1621*	/99
1F3	*ymdD*		Hypothetical protein	−	−	++	ND	*z1681*	667/1,158
2A4			Hypothetical protein	+	−	+	+	*z3062*	54/414
4F11	*ydeK*		Hypothetical lipoprotein	−	−	+	ND	*z2195*	339/4,032
6B2	*yneK*		Hypothetical protein	±	−	+	ND	*z2176*	398/789
2C3	IG		50 bp downstream of hypothetical protein *yebO*	+	−	±	ND	*3′ z2871*	/288
3E9	IG		18 bp downstream of hypothetical protein	−	−	±	ND	*3′ z2217*	/1,320
4C7	IG		43 bp upstream of hypothetical protein	−	−	+	ND	*5′ z2717*	/384
6H10	IG		120 bp upstream of possible chaperone	−	−	+	+	*5′ z2594*	/366
4B6			Unknown, no homologues in database	+	−−	+	+		/

[a] The site of transposon insertion relative to the predicted start codon; the O157:H7 strain EDL 933 homologue, where present; and in vitro test results are shown.
[b] The intensities of hybridization signals by using signature tags from the output pools recovered from feces (F) and mucosa (M) compared with those of the input pool were measured as follows: −−, absent; −, poorly represented; ±, somewhat down; +, present.
[c] Secretion of EspD as detected by Western blotting compared with that for the 193nal^R parent strain was determined as follows: −, absent; ±, somewhat reduced; +, wild-type levels; ++, elevated. ND, not determined.
[d] Homologous gene in O157:H7 strain EDL933 (49).
[e] IG, intergenic.

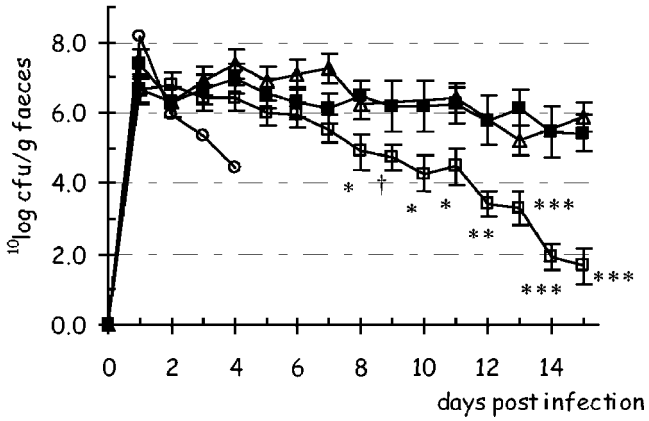

FIG. 3. Least-square means of fecal shedding (± standard errors of the means) of O26:H⁻ strain 193nal^R (■) in seven calves, 193nal^R*escN*::*Tn* mutant (○) in one calf, the 193nal^R *fimE*::*Tn* mutant (△) in three calves, and the 193nal^R *pssA*::*Tn* mutant (□) in four calves following oral challenge. †, $P < 0.1$; *, $P < 0.05$; **, $P < 0.001$; ***, $P < 0.0001$.

despite secreting normal levels of EspD. Sequencing of DNA flanking the *Tn* insertion generated a sequence with 96% nucleotide identity to *pkgA* of EDL933. In EDL933, *pkgA* lies adjacent to a defunct type III secretion system, ETT2. This gene encodes a homologue of phosphorylase-kinase-like glucoamylase (46), an enzyme widely present in eukaryotes and involved in glycogen metabolism. The fact that such enzymes are not widespread in prokaryotes, together with the close linkage of *pkgA* with ETT2, has led to the suggestion that PkgA is a type III secreted effector protein (46). PCR analysis of ETT2 in 193nal^R has revealed the presence of a deletion within ETT2 similar to that present in EPEC O111 (33), suggesting that ETT2 is nonfunctional in EHEC O26:H⁻ (data not shown).

Other surface structures. Type I fimbriae influence intestinal colonization by 193nal^R, as a mutation in *fimE* was detected in the STM screen (Table 1). The role of type I fimbriae, though, is not as one might predict. The expression of type I fimbriae is subject to phase variation, resulting in individual cells switching between a fimbriate and nonfimbriate state (56). This is due to the inversion of a 314-bp DNA segment located immediately upstream of the *fimA* gene, which encodes the

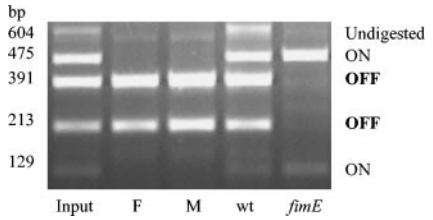

FIG. 4. Orientation of the *fim* switch in EHEC O26:H$^-$ strain 193nal^R, the 193nal^R *fimE*::*Tn* mutant, and input and output pools from an orally inoculated calf after amplifying the switch (604-bp band), followed by HinfI digestion. The ON orientation resulted in 475- and 129-bp bands; the OFF orientation resulted in 391- and 213-bp bands. F, feces; M, mucosa.

major subunit protein. A promoter residing in this phase switch drives the expression of the *fim* genes in the ON orientation but not when it is inverted. Two recombinases, FimB and FimE, mediate the inversion of the *fim* phase switch. FimB, although initially thought of as the "ON switch," was recently found to be able to carry out recombination in both directions, whereas FimE catalyzes phase switching from ON to OFF only (56). When grown in vitro, the parental 193nal^R strain consists of a mixed population, while the *fimE* mutant carries the *fim* switch predominantly in the ON position (Fig. 4). Both mutant and parent strains agglutinated yeast which could be inhibited with α-D-mannose. The *fimE* mutant, however, had the better ability to agglutinate and needed more α-D-mannose (>3%) to completely inhibit the agglutination (data not shown). The detection of a *fimE* mutant in the ON orientation in the STM screen would therefore suggest that expression of type I fimbriae is detrimental for intestinal colonization by EHEC O26:H$^-$.

To further investigate the role of type I fimbriae in intestinal colonization, we assessed expression of the fimbriae in 193nal^R both in vitro and following oral inoculation of calves. This was carried out by determining the orientation of the switch in the different bacterial populations (input and output pools). In the bacteria grown in LB broth in vitro, the switch was detected in both the ON and OFF orientations (Fig. 4, input). In contrast, in bacteria recovered from intestinal contents or mucosa, the switch was predominantly OFF (Fig. 4, F and M).

The *fimE* mutant was assessed for intestinal colonization in three calves. Surprisingly, the mutant was excreted in similar numbers as the parent strain (Fig. 3). The orientation of the *fim* switch was determined by colony PCR. In the inoculum, all colonies checked were in the ON position. On day 5, however, in the majority of the *fimE* colonies recovered from the feces, the switch was in the OFF orientation, indicating that the mutant still had the capability to invert the *fim* switch, as has been shown previously (56). All parent strain colonies recovered from the feces were in the OFF orientation. Taken together, our observations suggest there is a strong selection against type I fimbriation of EHEC in the bovine intestine.

A second EHEC O26:H$^-$ fimbrial operon was implicated in intestinal colonization. Mutant 4E3 had a *Tn* insertion in a region of the genome with 96% nucleotide identity to ORF *z2205* of EDL933. ORF *z2205* has 98% identity to *fimG* of *E. coli* K-12. FimG is a minor component of the type I pilus; associated with FimH, it plays a critical role in nucleating the formation of the adhesive tip of the fimbriae (27).

Expression of lipopolysaccharides is important for bacterial survival in the intestinal tract, and predictably, three mutants in the O antigen gene cluster of O26 (16) were detected in the STM screen. The *rmlB* and *fnl2* genes are responsible for the synthesis of nucleotide precursors for sugars, while the *wbuA* gene encodes a glycosyl transferase, which is highly specific for O26 (16). The *wbuA* mutant no longer showed agglutination with the O26-specific typing serum.

Cytotoxins. Four STM mutants were identified with insertions associated with genes encoding cytotoxins. Mutants with insertions in *pssA* were identified on two independent occasions in the screen (Table 1). *pssA* encodes a member of the secreted serine protease autotransporter of *Enterobacteriaceae* (SPATE) family (15). A mutant with a *Tn* insertion in *ehxA* was absent from the output pool collected from the mucosa; *ehxA* encodes enterohemolysin (54). Mutant 4B5 was shown to have a transposon insertion intergenic between *efa-1* and *st46* at 360 bp 5′ of *efa-1*. The *efa-1* gene is 99% identical to the EPEC *lifA* gene, which encodes the cytotoxin lymphostatin (28). Previous studies in our laboratory have implicated this gene in EHEC intestinal colonization (58). All four cytotoxin mutants were FAS positive and secreted EspD at levels comparable to those of the parent strain, demonstrating that the colonization defect was independent of the LEE-encoded type III secretion system.

In a separate calf oral challenge study, pure cultures of the *pssA* mutant (3E2, four calves) or the parent strain were used. The *pssA* mutant was excreted in similar numbers as the parent strain until day 6 postchallenge (Fig. 3). From day 8 onward, a significant ($P < 0.05$) reduction in shedding was observed.

Putative and unknown-function genes. One mutant was isolated with an insertion in a homologue of *evgS* (Table 1). EvgS is the sensor kinase of the EvgAS two-component sensory system that controls acid resistance genes in *E. coli* (37). Thus, the ability of EHEC to sense and respond to acid pH is likely to be important in determining the outcome of intestinal infection. Another mutant impaired in intestinal colonization, 3A6, was found to have an insertion three genes downstream of *evgS* in the opposite orientation. It has a *Tn* insertion in *yfdV*, a gene that encodes a putative receptor protein.

Many noncolonizing mutants have an insertion in loci likely to have genes involved in central intermediary metabolism and transport systems (Table 1). Also, a group of disrupted genes of unknown function was detected (Table 1). Nine mutants have insertions at loci not present in EDL933. One of these mutants, 4B6, has an insertion in a locus apparently unique to EHEC O26:H$^-$.

DISCUSSION

The emergence over the past 20 years of EHEC O157:H7 as a major zoonotic pathogen has led to considerable efforts to determine how this organism colonizes the intestinal tract and causes disease. Non-O157:H7 EHEC represents an emerging threat to animal and public health and welfare (3, 22). Therefore, we sought to compare and contrast the mechanisms of intestinal colonization of O157:H7 EDL933 with an isolate of serotype O26:H$^-$, associated with an outbreak of calf dysen-

tery. The present study shows that EHEC O157:H7 and O26:H$^-$ efficiently colonize bovine intestines, as indicated by prolonged high levels of fecal shedding compared with those of a commensal *E. coli* isolate. EHEC O26:H$^-$ was seen to interact closely with intestinal mucosa throughout the large intestines, whereas EHEC O157:H7 formed only sparse microcolonies at this site. Taken together with the finding that EHEC O157 and non-O157 EHEC differ in their tropism for the terminal rectum of calves (44), these observations indicate that these organisms may colonize the intestines by distinct mechanisms.

The infection model utilized in the screen was developed specifically to ensure reproducible intestinal colonization as required for the genetic analysis of bacterial colonization factors. Thus, it may not necessarily reflect colonization events that occur in other ages of animals and after extended periods of colonization as has been reported to occur in the field (44).

STM has proven to be a powerful method for screening large numbers of EHEC O26:H$^-$ mutants in vivo. Early in the screening, colonization-defective mutants with transposon insertions within the LEE were identified. This result indicated that the STM screen was able to reproducibly detect noncolonizing mutants, since both rabbit enteropathogenic *E. coli* (1, 35) and *C. rodentium* (13, 42) also require LEE genes for the formation of AE lesions and efficient intestinal colonization in vivo.

Our results show that disruption of the LEE-encoded type III secretion system affects colonization. This is consistent with the finding that intimin is an important colonization factor in ruminants (10, 12). However, the role of intimin-Tir interactions in intestinal colonization remains unclear, as intimin can also bind to β1 integrins (19) and to cell-surface-localized nucleolin (55). We have recently observed that the LEE-encoded type III secretion system is also required for the colonization of calves by EHEC O157:H7 (18), indicating that it plays an important role in the colonization of the bovine intestine by EHEC. Furthermore, these data imply that the combined activities of type III secreted proteins are crucial to the outcome of infection.

Fimbriae are cell surface appendages widely used by bacteria for attachment to host tissues during infection. Type I pili are heteropolymeric mannose-binding fibers produced by all members of the *Enterobacteriaceae* family (27). Type I fimbriae are important in the pathogenesis of urinary tract infections caused by uropathogenic *E. coli* (5, 41). In addition, *E. coli* K1, which is associated with extraintestinal infections, requires type I fimbriae to colonize the intestines of mice (36). In pig intestines, type I fimbriae were important for attachment to enterocytes and promoted intestinal colonization of *S. enterica* serovar Typhimurium (2). However, they were not required for phagocytosis or intracellular survival. The role of type I fimbriae in the pathogenesis of intestinal infections remains relatively unclear.

Recently, it was shown that EHEC O157:H7, but not non-O157:H7, strains carry a 16-bp deletion within the regulatory region of *fimA*, preventing the expression of type I fimbriae (51). Differential expression of these fimbriae by the EHEC O157:H7 and O26:H$^-$ strains used in this study could have led to the different pattern of intestinal colonization observed. However, enhanced expression of type I fimbriae, as seen with the *fimE* mutant, seems to be detrimental for the persistence of EHEC O26:H$^-$ in vivo. This may be through the promotion of undesirable interactions with the host. The *fimE* mutation may promote EHEC interactions with neutrophils, which may lead to more rapid clearance of the mutant from the intestinal tract. Type I fimbriae activate both neutrophils and mast cells and modulate phagocytosis in mice (34). Furthermore, type I fimbriated cells were at a disadvantage in initiating the colonization of streptomycin-treated mouse large intestine because of difficulty in entering the mucus layer of the intestine as rapidly as nonfimbriated cells (38).

A noncolonizing mutant with the insertion in a region of the chromosome showing homology to a second type I fimbria-like operon (ORFs *z2199* through *z2206* of EDL933) was identified. If the operon were functional in EHEC O26:H$^-$, the insertion would disrupt expression of a FimG-like protein. A mutant with an insertion in *z2203* was recently identified in an STM screen for EHEC O157:H7 genes required for the colonization of calves, and a defined mutant lacking the putative major fimbrial subunit of this locus was observed to be attenuated in calves (18). It remains unclear, however, if the genes at this locus encode a fimbria per se or if they contribute to the composition or surface expression of fimbriae encoded at distal loci. Further studies are required to assess the role of this conserved EHEC colonization factor.

The role of cytotoxins in EHEC colonization has been the subject of little attention to date. This may be due to the report that curing of the large plasmid of *E. coli* O157:H7, which was subsequently shown to encode enterohemolysin (EhxA) and EspP, the O157 homologue of PssA, had no effect on the pathogenesis of EHEC O157:H7 in gnotobiotic piglets (59). Enterohemolysin and the genetically and serologically unrelated alpha-hemolysin are members of the RTX family of toxins, associated with the ATP-dependent HlyB-HlyD-TolC secretion system through which toxin secretion occurs. EhxA exhibits pore-forming activity (54), yet the precise role of EhxA during infection is still not known. A role for EhxA in pathogenesis is likely, as the majority of EHEC cells isolated from humans and calves were shown to express functional hemolysin (6, 53) and antibody responses have been detected in convalescent patients (26). Our screening showing that an insertion within *ehxA* influences intestinal colonization further indicates that this putative virulence factor may play a role in pathogenesis, at least in EHEC O26:H$^-$.

The *pssA* gene encodes a 104-kDa protein that has serine protease activity and is cytotoxic for VERO cells (15). The prevalence of *pssA* on the large plasmid in EHEC O26:H$^-$ and O26:H11 is about 60% (8). PssA and the EHEC O157:H7 homologue EspP belong to the SPATE group of proteins (24). These include EspC of EPEC, Tsh of avian pathogenic *E. coli*, and Pet of enteroaggregative *E. coli*. To date, few studies using isogenic mutants have ascribed a role in pathogenesis to SPATEs. The discovery of a *Tn* insertion within *pssA* is the first direct evidence demonstrating a role for a SPATE protein in the intestinal colonization of calves by EHEC. Despite sharing a motif common to certain serine proteases, the cleavage profiles of oligopeptides for different SPATEs are unique and these proteins differ in their toxicities for cultured cells (17). Together, these observations suggest that SPATEs have different roles in *E. coli* pathogenesis.

Apparently, different EHEC serotypes differ in their reliance on cytotoxins to colonize the intestines of calves. Of 62 characterized O26:H$^-$ mutants, 4 were associated with genes encoding cytotoxins (*pssA* 2x, *ehxA*, and *efa-1*). Somewhat surprisingly in a related study using STM to identify the O157:H7 genes involved in colonizing the intestines of calves, of 79 characterized mutants, none were identified encoding cytotoxins (18). This difference may be a reflection on the nature of the interaction between EHEC O157:H7 and O26:H$^-$ strains with bovine intestines. EHEC O26:H$^-$, unlike O157:H7, interacts intimately in large numbers with intestinal epithelial cells (Fig. 2). This is consistent with the greater enteropathogenicity of EHEC O26:H$^-$ than that of O157:H7 for calves (52). Enteropathogenesis is associated with acute intestinal inflammatory responses; thus, EHEC O26:H$^-$ is likely to have to resist the bactericidal activities of inflammatory cells during intestinal colonization. This is likely to require the utilization of various cytotoxins to inactivate the host responses if productive infection is to result.

Several genes with an unknown function were identified as having a role in the colonization or survival of EHEC O26:H$^-$ in vivo. Transcriptome and proteome studies are necessary to facilitate the characterization of these colonization factors.

To conclude, our studies have shown that different EHEC serotypes colonize calves with distinct tropisms within the intestinal tract. We have shown that EHEC O26:H$^-$ utilizes type III secreted proteins and cytotoxins but apparently not classical type I fimbriae to colonize the intestines of calves. These observations have important implications for the development of vaccines to eliminate EHEC from animal reservoirs.

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