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## Detection and Characterization of the Fimbrial *sfp* Cluster in Enterohemorrhagic *Escherichia coli* O165:H25/NM Isolates from Humans and Cattle<sup>∇</sup>

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The *sfp* cluster, encoding Sfp fimbriae and located in the large plasmid of sorbitol-fermenting (SF) enterohemorrhagic *Escherichia coli* (EHEC) O157 (pSFO157), has been considered a unique characteristic of this organism. We discovered and then characterized the *sfp* cluster in EHEC O165:H25/NM (nonmotile) isolates of human and bovine origin. All seven strains investigated harbored a complete *sfp* cluster (carrying *sfpA*, *sfpH*, *sfpC*, *sfpD*, *sfpJ*, *sfpF*, and *sfpG*) of 6,838 bp with >99% nucleotide sequence homology to the *sfp* cluster of SF EHEC O157:NM. The *sfp* cluster in EHEC O165:H25/NM strains was located in an ~80-kb (six strains) or ~120-kb (one strain) plasmid which differed in structure, virulence genes, and *sfp* flanks from pSFO157. All O165:H25/NM strains belonged to the same multilocus sequence type (ST119) and were only distantly phylogenetically related to SF EHEC O157:NM (ST11). The highly conserved *sfp* cluster in different clonal backgrounds suggests that this segment was acquired independently by EHEC O165:H25 and SF EHEC O157:NM. Its presence in an additional EHEC serotype extends the diagnostic utility of PCR targeting *sfpA* as an easy and efficient approach to seek EHEC in patients' stools. The reasons for the convergence of pathogenic EHEC strains on a suite of virulence loci remain unknown.

*Escherichia coli* O157:H7 is the most commonly isolated serotype of enterohemorrhagic *E. coli* (EHEC) worldwide (29, 48). However, an increasing number of non-O157:H7 EHEC serotypes have been isolated from patients (3, 5, 9, 19, 27, 28, 29, 31, 32, 33, 51). Different serotypes possess various, often serotype-specific, combinations of virulence genes (5, 46, 49, 55) within genomic islands, bacteriophages, or large plasmids (4, 10, 11, 12, 13, 15, 16, 30, 37, 38, 39). Allelic variations of some genes, such as *eae*, encoding the adhesin intimin, and of course *stx*, encoding Shiga toxin (Stx), are present in most EHEC strains (3, 5, 9). However, other genes are restricted to particular serotypes. Such genes include, for example, the *lpf* operon (encoding long polar fimbriae) in O island (OI) 154 of *E. coli* O157:H7 EDL933 (50), which has been found only in EHEC O157:H7/NM (nonmotile) isolates and their progenitor, *E. coli* O55:H7 (5, 49, 50), and the *sfp* gene cluster, which we recently proposed to be restricted to the large plasmid of sorbitol-fermenting (SF) EHEC O157:NM, pSFO157 (11, 12). The *sfp* cluster is inserted into the region of pSFO157 where *katP* and *espP* (encoding a catalase-peroxidase and a serine protease, respectively) reside in the large plasmid of EHEC O157:H7, pO157 (13). The cluster is flanked by insertion sequences and an origin of plasmid replication (11, 12), indicat-

ing that horizontal transfer gave rise to its presence in SF EHEC O157:NM.

The *sfp* cluster (carrying *sfpA*, *sfpH*, *sfpC*, *sfpD*, *sfpJ*, *sfpF*, and *sfpG*) encodes Sfp fimbriae that mediate mannose-resistant hemagglutination (11) and possibly also adherence of SF EHEC O157:NM organisms to human intestinal epithelial cells (34). The absence of this locus in thousands of Stx-producing *E. coli* strains belonging to many different serotypes led us to assume that the *sfp* cluster is unique to SF EHEC O157:NM (23). However, using PCR to target *sfpA* (encoding the major fimbrial subunit) in stool samples (23), we unexpectedly isolated an *sfpA*-positive EHEC strain of serotype O165:H25 from a patient with hemolytic-uremic syndrome (HUS). Here we report the extended analysis of this serotype, focusing on conservation of the *sfp* cluster.

### MATERIALS AND METHODS

**Bacterial strains.** *sfpA*-positive EHEC O165:H25 strain 820/08 from a patient with HUS was isolated at the National Consulting Laboratory for HUS, Münster, Germany, during routine diagnostic efforts by use of a published protocol (23). Additionally, five EHEC O165:H25/NM strains, four from epidemiologically unrelated patients with diarrhea and one from a healthy bull (Table 1), were from the collection of the Robert Koch Institute, Wernigerode, Germany. Three of these strains were described previously (25). EHEC O165:H25 strain MT52 (Table 1) was from a patient with bloody diarrhea in Montana (27). SF EHEC O157:NM HUS isolate 3072/96, in which the *sfp* cluster was originally identified (11), and EHEC O157:H7 strain EDL933 (13, 35) were used as positive and negative controls, respectively.

**Phenotyping.** Isolates were confirmed as *E. coli* (API 20 E; bioMérieux, Nürtingen, Germany) and serotyped (27, 40). Fermentation of sorbitol was determined on sorbitol MacConkey agar (SMAC),  $\beta$ -D-glucuronidase activity was assessed using nutrient agar with 4-methylumbelliferyl- $\beta$ -D-glucuronide

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TABLE 1. EHEC O165:H25/NM strains and controls investigated in this study, with plasmid characteristics

Strain no.	Yr, country of isolation <sup>a</sup>	Clinical diagnosis <sup>b</sup> or source	Serotype <sup>c</sup>	<i>fliC</i> RFLP type	Plasmid characteristics					
					Plasmid profile <sup>d</sup>	Southern blot hybridization with probe <sup>e</sup>				
						<i>sfpA</i>	EHEC <i>hlyA</i>	<i>espP</i> <sup>f</sup>	<i>katP</i>	<i>etpD</i>
820/08	2008, G	HUS	O165:H25	H25	80	80	80	80	80	—
98-08419-1	1998, G	D	O165:NM	H25	80, 120	80	80	80	80	—
99-02258	1999, G	D	O165:H25	H25	80, 120	80	80	80	80	—
02-11228	2002, G	D	O165:H25	H25	80, 120	120	80, 120	80	80, 120	—
04-07734	2004, G	D	O165:H25	H25	80	80	80	80	80	—
MT52	2000, US	BD	O165:H25	H25	80	80	80	80	80	—
00-09087	2000, G	Cattle	O165:H25	H25	60, 80	80	80	80	80	—
3072/96	1996, G	HUS	O157:NM	H7	121	121	121	—	—	121
EDL933	1982, US	Hamburger	O157:H7	H7	92	—	92	92	92	92

<sup>a</sup> G, Germany; US, United States.

<sup>b</sup> HUS, hemolytic-uremic syndrome (defined as the triad of hemolytic anemia, thrombocytopenia, and acute renal failure) (48); BD, bloody diarrhea; D, diarrhea.

<sup>c</sup> NM, nonmotile.

<sup>d</sup> Sizes of plasmids (in kb). The sizes of plasmids in strains 3072/96 and EDL933 are based on published sequences (GenBank accession no. AF401292 and AF074613, respectively) (12, 13); the sizes of the other plasmids were estimated according to their mobility by agarose gel electrophoresis (Fig. 2A).

<sup>e</sup> The *sfpA*, EHEC *hlyA*, *espP*, *katP*, and *etpD* genes encode the major subunit of Sfp fimbriae, EHEC hemolysin, a serine protease, a catalase-peroxidase, and a type II secretion system, respectively (12, 13). The data are sizes (in kb) of plasmids that hybridized with the respective probes. —, no signal. The presence or absence of the respective genes was confirmed by PCR.

<sup>f</sup> All EHEC O165:H25/NM strains contain *espP* subtype  $\delta$ , whereas strain EDL933 contains *espP* subtype  $\alpha$ , as demonstrated using the *espP* subtyping scheme (8).

(Becton Dickinson, Sparks, MD), EHEC hemolysin production was identified using enterohemolysin agar (Sifin, Berlin, Germany), and production of  $\alpha$ -hemolysin was sought with Columbia blood agar (Heipha, Heidelberg, Germany). Resistance to tellurite was determined by assessing the ability of strains to grow on cefixime-tellurite SMAC (CT-SMAC). Stx production was determined using a Vero cell assay (3).

**Analysis of the *sfp* cluster.** The presence of the complete *sfp* cluster was sought using concatenated PCRs in which each *sfp* gene and linkages between contiguous genes were amplified (Fig. 1 and Table 2). Purified PCR products (PCR purification kit; Qiagen, Hilden, Germany) were sequenced using an automated ABI Prism 3130xl genetic analyzer and an ABI Prism BigDye Terminator ready reaction cycle sequencing kit (version 3.1; Applied Biosystems, Darmstadt, Ger-

many). Sequences were analyzed with Ridom TraceEditPro software (Ridom GmbH, Würzburg, Germany), and homology was sought using the EMBL-GenBank database (<http://www.ncbi.nlm.nih.gov/BLAST>).

**Genotypic characterization.** Putative virulence genes, *ter* genes encoding tellurite resistance (Table 3), and plasmid genes (Table 1) were sought using PCR (1, 2, 5, 8, 11, 18, 30, 46, 49). *fliC*, encoding the flagellin subunit, and *aeae*, *espP*, and *stx* genes were subtyped (3, 8, 46, 54, 55). The *stx*<sub>2a</sub> activatable allele (3, 56) was sought using a specific PCR (56).

**Plasmid analysis.** Plasmid profiles were determined (41), and plasmid restriction fragment length polymorphism (RFLP) patterns were produced using plasmid-extracted DNA (Plasmid Midi kit; Qiagen) digested with EcoRI (New England Biolabs, Frankfurt, Germany) and separated in a 0.6% agarose gel. For

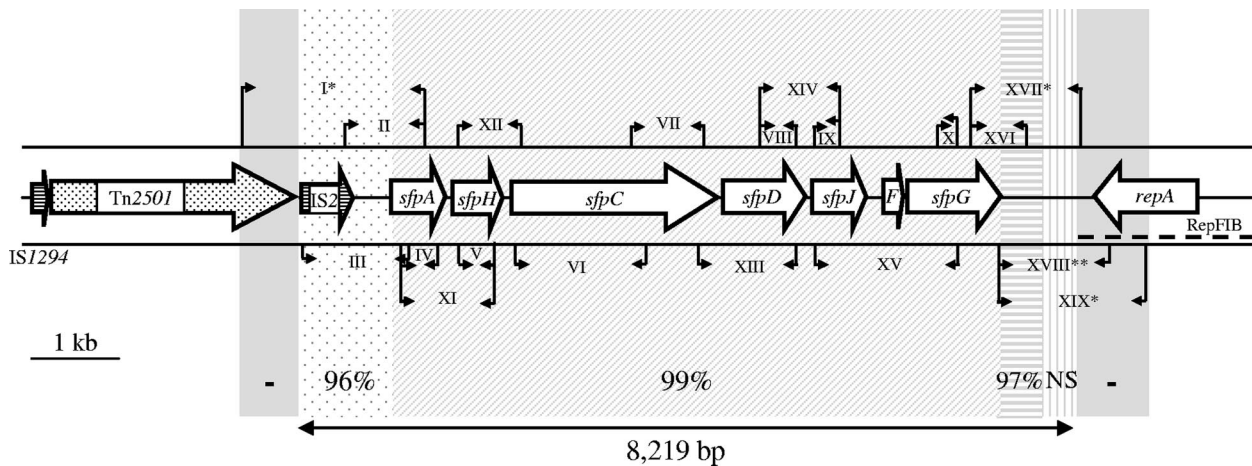


FIG. 1. Physical map of the *sfp* cluster and its flanks in SF EHEC O157:NM strain 3072/96 and PCR strategy used for analysis of the *sfp* cluster in EHEC O165:H25/NM. The large arrows indicate *sfp* genes (*F*, *sfpF*) and genes adjacent to the *sfp* cluster in strain 3072/96. A transposon-like element is dotted, and insertion sequences are horizontally striped. The dashed line below *repA* depicts the RepFIB origin of replication of pSFO157. The small arrows indicate the positions of PCR primers (details for PCRs I to XIX are given in Table 2). In all but four PCRs, amplicons of identical sizes were obtained from each of seven EHEC O165 strains and strain 3072/96. In the remaining PCRs, no amplicons (\*) or amplicons of different sizes (\*\*) than those from strain 3072/96 (~600 bp instead of 1,343 bp) were elicited from EHEC O165 strains. The double arrow below the graph depicts the 8,219-bp plasmid region which was sequenced from EHEC O165:H25 strains 820/08 and 04-07734 and which comprises the *sfp* cluster (6,838 bp) and its upstream (828 bp) and downstream (553 bp) flanks. The numbers above this arrow indicate nucleotide sequence homologies of the corresponding regions between EHEC O165:H25 strains (which were 100% identical) and strain 3072/96. NS, no significant homology was found using BLAST algorithms (<http://www.ncbi.nlm.nih.gov/BLAST>). The gray regions (—) adjacent on both sides to the sequenced stretch were not detected in EHEC O165 by the PCR strategy used.

TABLE 2. PCR primers and conditions used for analysis of the *sfp* cluster and its flanking regions in EHEC O165:H25/NM

PCR no.	Primer <sup>a</sup>	Sequence (5'–3')	Target	PCR conditions <sup>b</sup>			PCR product size (bp)
				Denaturing	Annealing	Extension	
I	wprom-3 wprom-4	GCT GGA CGC CGG TGC TTA TT CCA GAT TGC CGC TCG CTT CAG	Tn2501- <i>sfpA</i>	94°C, 30 s	61°C, 60 s	72°C, 150 s	1,900
II	IS2-1 wprom-4	GTG ATA ACA AGC GTC TGG AAA CCA GAT TGC CGC TCG CTT CAG	IS2- <i>sfpA</i>	94°C, 30 s	57°C, 60 s	72°C, 60 s	867
III	IS2-3 sfpA-2	CGA TGA CTG GAA GGA TAG AGC CAG TTC CTT GAC ATC	IS2- <i>sfpA</i>	94°C, 30 s	50°C, 60 s	72°C, 90 s	1,161
IV	sfpA-U sfpA-L	AGC CAA GGC CAA GGG ATT ATT A TTA GCA ACA GCA GTG AAG TCT C	<i>sfpA</i>	94°C, 30 s	59°C, 60 s	72°C, 60 s	440
V	sfpH-1 sfpH-2	CCA TCG CCC TCC TTT CCT CT TCC AGC CCC TCT TCA TTT CC	<i>sfpH</i>	94°C, 30 s	58°C, 60 s	72°C, 60 s	425
VI	sfpC-5 sfpC-6	TAT GCG TTG TTG TTT TCC TCC CGT TTC TGG TAT TTC	<i>sfpC</i> 5' end	94°C, 30 s	55°C, 60 s	72°C, 150 s	1,643
VII	sfpC-1 sfpC-2	CGG GCA TCG GAC TAT TAC AC CCA CCA TCC CCA GTT CAC G	<i>sfpC</i> 3' end	94°C, 30s	58°C, 60 s	72°C, 60 s	787
VIII	sfpD-1 sfpD-2	CGC CAA TGA TAA CAA ACA G TAC CAC CAA TGC CAA TCA C	<i>sfpD</i>	94°C, 30 s	52°C, 60 s	72°C, 60 s	410
IX	sfpJ-1 sfpJ-2	ATG AGC AGG ACA ACA ACA GG TAT TTT CCG TTA TCC TCC AG	<i>sfpJ</i>	94°C, 30 s	52°C, 60 s	72°C, 60 s	251
X	sfpG-1 sfpG-2	CGT AGT GAC TTG GAT GCT G TTG CCA TCC ATT ACC ATT C	<i>sfpG</i>	94°C, 30s	52°C, 60s	72°C, 60s	267
XI	sfpA-U sfpH-2	AGC CAA GGC CAA GGG ATT ATT A TCC AGC CCC TCT TCA TTT CC	<i>sfpA-sfpH</i>	94°C, 30s	59°C, 60s	72°C, 60s	1,026
XII	sfpH-1 sfpC-3	CCA TCG CCC TCC TTT CCT CT CGC CAG CCA GGG AAA ACA ACA A	<i>sfpH-sfpC</i>	94°C, 30s	59°C, 60s	72°C, 60s	733
XIII	sfpC-4 sfpD-2	GTG GGG AGT TGG TGT AGT GA TAC CAC CAA TGC CAA TCA C	<i>sfpC-sfpD</i>	94°C, 30s	56°C, 60s	72°C, 60s	1,030
XIV	sfpD-1 sfpJ-2	CGC CAA TGA TAA CAA ACA G TAT TTT CCG TTA TCC TCC AG	<i>sfpD-sfpJ</i>	94°C, 30s	52°C, 60s	72°C, 60s	875
XV	sfpJ-1 sfpG-2	ATG AGC AGG ACA ACA ACA GG TTG CCA TCC ATT ACC ATT C	<i>sfpJ-sfpG</i>	94°C, 30s	52°C, 60s	72°C, 150s	1,748
XVI	sfpG-3 Gfla-1	GCA TAA CAA TAA ACT CCA AA TGA CAA GCA AAC TGA TTA CC	<i>sfpG</i> Flank 1	94°C, 30 s	48°C, 60 s	72°C, 60 s	588
XVII	sfpG-3 Gfla-3	GCA TAA CAA TAA ACT CCA AA TAT TGC TCC CTT CAT AAA A	<i>sfpG</i> Flank 2	94°C, 30 s	50°C, 60 s	72°C, 120 s	1,325
XVIII	sfpG-5 Gfla-4	GGA TCG TAA TGC TGT TTC A AAG CAC CGA ATG ATG AGA G	<i>sfpG-repA</i>	94°C, 30 s	51°C, 60 s	72°C, 150 s	1,343
XIX	sfpG-5 Gfla-5	GGA TCG TAA TGC TGT TTC A TAC CGT GAT CTC CTT TTC C	<i>sfpG-repA</i>	94°C, 30 s	51°C, 60 s	72°C, 180 s	1,768

<sup>a</sup> The primers were derived from the respective genes of the *sfp* cluster and from the *sfp* flanking regions in SF EHEC O157:NM strain 3072/96 (GenBank accession no. AF401292). Except for primer pairs wprom-3/wprom-4 and sfpA-U/sfpA-L, which were described previously (11), all primers were designed for this study.

<sup>b</sup> All PCRs included 30 cycles as given in the table, preceded by an initial denaturation (94°C, 5 min) and followed by a final extension (72°C, 5 min).

Southern hybridization, undigested or EcoRI-digested plasmid DNA was transferred to a nylon membrane (Roche Molecular Biochemicals, Mannheim, Germany) and hybridized with digoxigenin-labeled (DIG High Prime kit; Roche Molecular Biochemicals) *sfpA*, EHEC *hlyA*, *katP*, *espP*, and *etpD* probes. The *sfpA* probe was derived from SF EHEC O157:NM strain 3072/96 by PCR with primers sfpA-U and sfpA-L (Table 2). All other probes were derived from *E. coli* O157:H7 strain EDL933, using primers HlyA1 and HlyA4 (EHEC *hlyA*), *esp-A* and *esp-B* (*espP*), D1 and D13R (*etpD*) (46), and *kat-1* (5'-GGCGAAGAGA AGATGACTG-3') and *kat-2* (5'-GCCACAGTCTCTCATCATC-3') (*katP*). Labeled probes were detected using a DIG luminescence detection kit (Roche Molecular Biochemicals).

**Multilocus sequence typing (MLST) and PCR phylogrouping.** Internal regions of seven housekeeping genes were sequenced (4, 53) to assign alleles and sequence types (ST) (<http://web.mpiib-berlin.mpg.de/mlst/dbs/Ecoli>). Relationships between strains harboring the *sfp* cluster were characterized using the eBURST algorithm (21), which groups strains sharing six or more identical alleles into the same clonal complex (CC). Similarities were calculated using MEGA software v. 4.0 (47), and a minimum spanning tree was generated with SeqSphere software v. 0.7 beta (Ridom GmbH). In addition, isolates were classified into *E. coli* reference phylogenetic groups A, B1, B2, and D (43), using PCR (14).

**Strain accession number.** The EHEC O165:H25 HUS isolate 820/08 characterized in this study is included in our recently created HUSEC reference collection (33; [www.ehec.org](http://www.ehec.org)) as HUSEC042.

**Nucleotide sequence accession numbers.** The 6,838-bp *sfp* clusters and their 828-bp upstream and 553-bp downstream flanks in the large plasmids of O165:H25 strains 820/08 and 04-07734 were deposited in the EMBL-GenBank database under accession no. EU980314 and EU980315, respectively.

## RESULTS

**EHEC O165:H25/NM strains contain a complete *sfp* cluster.** All seven EHEC O165:H25/NM isolates yielded amplicons of the same sizes as those elicited from the control SF EHEC O157:NM strain 3072/96 in all PCRs targeting *sfp* genes and their links (Fig. 1). This demonstrates the resemblance between the *sfp* clusters of EHEC O165 and SF O157 strains.

**The *sfp* cluster is conserved in EHEC O165:H25/NM and SF EHEC O157:NM.** The sequences of the *sfp* clusters (6,838 bp) in EHEC O165:H25 strains 820/08 and 04-07734 (chosen to represent severe and mild disease phenotypes, respectively) were identical to each other and >99% identical to the *sfp* cluster of SF EHEC O157:NM strain 3072/96 (GenBank accession no. AF401292). The 23 nucleotide differences between

TABLE 3. Characteristics of EHEC O165:H25/NM and O157:NM strains harboring the *sfp* cluster

Characteristic <sup>a</sup>	Presence of gene, value, or description	
	O165:H25/NM (n = 7) <sup>b</sup>	O157:NM (3072/96)
<i>stx</i> genotype	<i>stx</i> <sub>2</sub> plus <i>stx</i> <sub>2c</sub> <sup>c</sup>	<i>stx</i> <sub>2</sub>
<i>cdt</i> <sup>d</sup>	–	+
<i>eae</i> type	ε	γ
<i>lpfA</i> <sub>O157/O113</sub>	–	+
<i>lpfA</i> <sub>O113</sub>	+	–
OI 122 ( <i>efa1/sen/nleE/nleB/pagC</i> ) <sup>e</sup>	+ / + / + / + / –	+ / + / + / + / +
<i>ter</i> cluster <sup>f</sup>	–	–
Sorbitol fermentation	– / + <sup>g</sup>	+
β-D-Glucuronidase	+	+
Growth on CT-SMAC	–	– <sup>h</sup>
EHEC hemolysin production	+	– <sup>h</sup>
α-Hemolysin production	–	–
Shiga toxin titer <sup>i</sup>	256–1,024	512
Phylogenetic group	A	D

<sup>a</sup> The genes mentioned encode the following proteins or phenotypes: *stx*, Shiga toxin; *cdt*, cytolethal distending toxin; *eae*, intimin; *lpfA*<sub>O113</sub> and *lpfA*<sub>O157/O113</sub>, major fimbrial subunits of long polar fimbriae of EHEC O113 and EHEC O157, respectively, encoded on OI 154; *efa1*, EHEC factor for adherence; *sen*, homologue of *Shigella flexneri* enterotoxin 2; *nleE* and *nleB*, non-locus-of-enterocyte-effacement effector proteins NleE and NleB, respectively; *pagC*, homologue of PagC protein of *Salmonella enterica* serovar Typhimurium; *ter* cluster, tellurite resistance. +, the characteristic is present; –, the characteristic is absent.

<sup>b</sup> All seven EHEC O165:H25/NM strains had identical characteristics, except for sorbitol fermentation (see below).

<sup>c</sup> One strain (bovine isolate 00-09087) lost *stx* before it could be subtyped.

<sup>d</sup> *cdt* I, *cdt* II, *cdt* III, *cdt* IV, and *cdt* V genes were targeted (1); strain 3072/96 contains the *cdt* V cluster.

<sup>e</sup> A complete OI 122 which contains all genes representative of its three modules (*efa1*, *sen*, *nleE*, *nleB*, and *pagC*) (30) was present in EHEC O157, whereas all EHEC O165 strains contained OI 122 without *pagC* (incomplete OI 122) (30).

<sup>f</sup> All *ter* genes (*terZ*, *terA*, *terB*, *terC*, *terD*, *terE*, and *terF*) were absent.

<sup>g</sup> All six EHEC O165:H25/NM strains from Germany are non-SF, whereas the U.S. strain MT52 is SF.

<sup>h</sup> The EHEC *hlyA* gene is present but not expressed.

<sup>i</sup> The highest dilution of a culture supernatant which caused cytotoxicity in 50% of the Vero cell monolayer after 3 days of incubation. The range of Stx titers for the seven EHEC O165:H25/NM strains is shown.

the *sfp* genes of EHEC O165:H25 and strain 3072/96 consisted of 10 nonsynonymous and 13 synonymous changes.

#### *sfp* clusters in EHEC O165:H25/NM are in large plasmids.

To determine if the *sfp* cluster in EHEC O165 is located on a plasmid, plasmids were isolated from the seven EHEC O165:H25/NM strains and hybridized with the *sfpA* probe. All strains contained a plasmid of ~80 kb, either alone or with a larger (~120 kb) or smaller (~60 kb) plasmid (Fig. 2A; Table 1). For six strains, the *sfpA* probe hybridized to the 80-kb plasmid (Fig. 2B, lanes 1, 4, and 6 to 8; Table 1), and for one strain, the probe bound to a 120-kb plasmid (Fig. 2B, lane 5). The latter strain was the only one in which the size of the *sfp*-harboring plasmid was comparable to that in SF EHEC O157:NM strain 3072/96 (Fig. 2B, lane 2).

**Comparison of *sfp*-harboring plasmids of EHEC O165:H25/NM strains and pSFO157.** *sfp*-harboring plasmids from EHEC O165:H25/NM strains (pO165) were further hybridized with probes representing a panel of putative virulence plasmid-carried genes of *E. coli* O157:H7 (EHEC *hlyA*, *espP*, *katP*, and *etpD*) (Table 1). In each instance where the *sfp* cluster was in the 80-kb plasmid (Fig. 2B, lanes 1, 4, and 6 to 8; Table 1), the

same plasmid also hybridized with the EHEC *hlyA*, *espP*, and *katP* probes (Table 1). In contrast, in the only strain that contained the *sfp* cluster in the 120-kb plasmid (Fig. 2B, lane 5), the same plasmid also contained EHEC *hlyA* and *katP* sequences, but *espP* was located in the second (80-kb) plasmid (Table 1); the latter plasmid also harbored additional copies of EHEC *hlyA* and *katP* (Table 1). None of the pO165 plasmids hybridized with the *etpD* probe (Table 1). In contrast to pO165, and in accordance with its sequence (GenBank accession no. AF401292), the 121-kb pSFO157 plasmid from strain 3072/96 hybridized with EHEC *hlyA* and *etpD* but not with the *katP* and *espP* probes (Table 1). Thus, pO165 and pSFO157 plasmids differ by gene composition in addition to (in most cases) size.

**Plasmid RFLP and *sfpA* Southern blot hybridization.** For three EHEC O165:H25 strains that contained a single plasmid (Table 1), the plasmid RFLP patterns of the two German strains were identical and differed from that of the U.S. strain; moreover, all three EHEC O165:H25 strains differed in plasmid RFLP pattern from strain 3072/96. For EHEC O165, the *sfpA* probe hybridized to either an ~50-kb (both German strains) or ~15-kb (MT52) fragment of plasmid DNA, whereas in strain 3072/96, an ~11-kb DNA fragment reacted with the probe (data not shown).

***sfp*-flanking regions in EHEC O165:H25/NM.** A fragment of the insertion sequence IS2, but not a transposon-like sequence (Tn2501), is located upstream of the *sfp* cluster in EHEC O165 strains (Fig. 1). Furthermore, a region homologous to the RepFIB origin of replication of pSFO157, which starts 686 bp downstream of *sfpG* in strain 3072/96 and includes an open reading frame for the replication protein RepA (11) (GenBank accession no. AF401292), was not found at this position in EHEC O165:H25/NM (Fig. 1). In these strains, a 205-bp fragment of the insertion sequence IS1 is located 333 bp downstream of *sfpG* (GenBank accession no. EU980314 and EU980315), displaying no significant homology to this region in pSFO157 (Fig. 1).

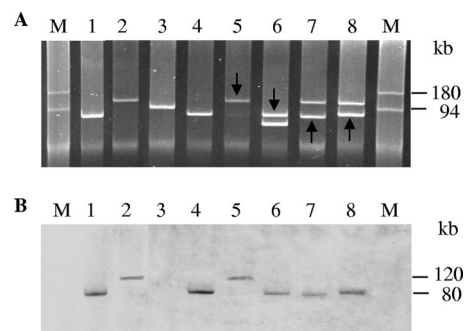


FIG. 2. Plasmid profiles (A) and plasmid hybridization with the *sfpA* probe (B) of EHEC O165 and control strains. In lanes 1 and 4 to 8, the following EHEC O165:H25/NM strains are shown: lanes 1, 820/08; lanes 4, 04-07734; lanes 5, 02-11228; lanes 6, 00-09087; lanes 7, 99-02258; and lanes 8, 98-08419-1. In lanes 2 and 3, SF EHEC O157:NM strain 3072/96 and EHEC O157:H7 strain EDL933, respectively, are shown for comparison. For strains which contained two plasmids, the plasmid which hybridized with the *sfpA* probe is marked by an arrow (A); the sizes of the *sfpA*-hybridizing plasmids are indicated in panel B. M, molecular size marker (DNAs of plasmids R27 [180 kb] and R100 [94 kb]) (45; <http://www.biotech.bham.ac.uk/Plasmids/default.htm>).

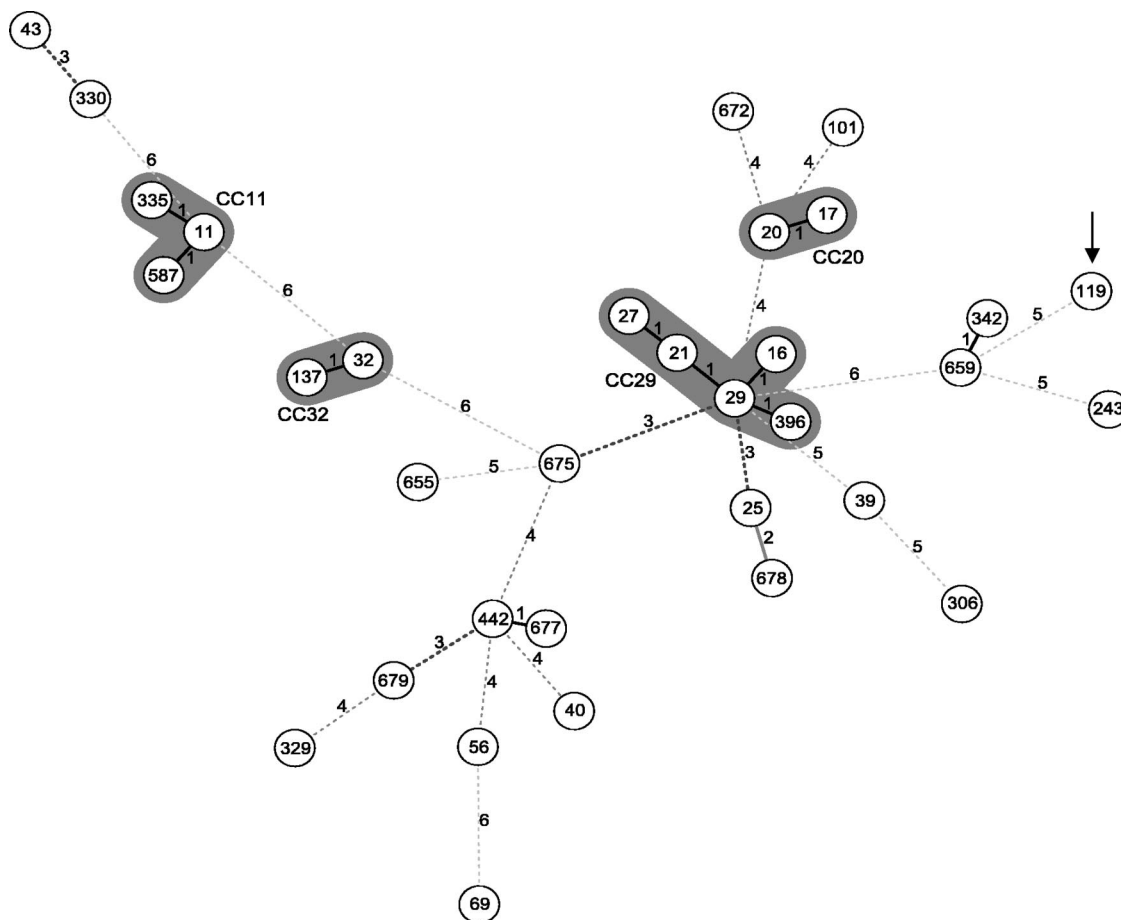


FIG. 3. Minimum spanning tree based on MLST of the *sfp*-positive EHEC O165:H25/NM strains (ST119; depicted by arrow) in comparison to the HUSEC collection (33) and *Shigella dysenteriae* strain M1354 as an outgroup (ST243) (data are from <http://web.mpiib-berlin.mpg.de/mlst/dbs/Ecoli>). Each circle represents a unique ST (with the designation given in the circle). The numbers on the connecting lines display the numbers of differing alleles. Clonal complexes (CC) of the major EHEC serogroups (33) are highlighted in gray.

**Virulence genes and phenotypes of EHEC O165:H25/NM strains containing an *sfp* cluster.** In addition to the *sfp* cluster and other plasmid genes (EHEC *hlyA*, *espP*, and *katP*) (Table 1), the EHEC O165:H25/NM strains also shared chromosomal virulence loci and displayed, except for sorbitol fermentation by the U.S. isolate MT52, identical phenotypes (Table 3). However, they differed from SF EHEC O157:NM with respect to most of these characteristics (Table 3).

**MLST analysis and phylogeny PCR.** The phylogenetic relationships between EHEC O165:H25/NM strains and representative HUS-associated EHEC strains (HUSEC collection) (33) are shown in Fig. 3. All EHEC O165:H25/NM strains with the *sfp* cluster belonged to the same sequence type (ST119), which is quite distinct from ST11 of SF EHEC O157:NM strain 3072/96. Furthermore, EHEC O165:H25/NM strains share only two of the seven loci with the next most closely related organisms (ST342 and ST659). At the nucleotide level, ST119 and ST11 differ in 48 of the 3,423 bp analyzed by MLST in the seven housekeeping genes (1.40% pairwise distance). In contrast, the overall average pairwise distance within the HUSEC collection is only 0.89%. The *sfp*-positive EHEC O165:H25/NM isolates differ at only 24 sites (0.70%) from *Shigella dysenteriae* strain M1354 (ST243) (<http://web.mpiib-berlin.mpg.de/mlst>

[/dbs/Ecoli](http://web.mpiib-berlin.mpg.de/mlst/dbs/Ecoli)), which was used as an outgroup for the minimum spanning tree (Fig. 3).

Phylogenetic grouping (43) further supports the distant relationship between the O165 and O157 EHEC strains carrying *sfp* clusters. Whereas SF EHEC O157:NM strains belong to *E. coli* reference group D, all seven O165:H25/NM strains were classified as group A (Table 3). These groups represent the two extremes of the *E. coli* population (43, 53).

## DISCUSSION

We identified the fimbrial *sfp* operon, which we previously proposed to be unique to SF *E. coli* O157:NM (23), in an additional EHEC serotype, O165:H25. Despite their host cells' distant relatedness, the *sfp* cluster is highly conserved in these two serotypes and resides on large plasmids. However, the *sfp*-harboring plasmids in the two serotypes differ in size, structure, gene composition, and position of the *sfp* cluster, as well as in the sequences flanking the *sfp* genes. Our data have diagnostic, epidemiological, and evolutionary implications.

EHEC O165 strains cause HUS and bloody diarrhea (7, 9, 17, 20, 22, 27, 28, 31, 44, 52). The need to reliably identify such strains is therefore obvious. Their possession of the *sfp* cluster



offers an additional target, besides *stx/Stx*, to detect them in patients' stools. In work that led to the identification of the *sfp* cluster in EHEC O165, we were initially stymied by the presence of *sfpA* and the absence of *rfb*<sub>O157</sub>, which then prompted extensive efforts to isolate the strain of interest and its subsequent characterization. Thus, the PCR detection of *sfpA* and the inability to detect *rfb*<sub>O157</sub> provided the impetus to identify an infecting yet unexpected pathogen in the primary stool culture. A systematic pursuit of such findings by isolation of the PCR-positive strains might lead in the future to identification of other *E. coli* serotypes that harbor the *sfp* cluster. An additional diagnostically useful marker of EHEC O165:H25/NM strains is that they mostly fail to ferment sorbitol after overnight incubation on SMAC (Table 3). This unusual phenotype, which is generally restricted to EHEC O157:H7 (29, 48) and other select EHEC isolates (44; www.ehec.org) and which was also reported for an EHEC O165:NM strain isolated from an HUS patient in Brazil (17), can assist in the isolation of such strains from primary cultures. However, in contrast to EHEC O157:H7, the EHEC O165:H25/NM strains produce  $\beta$ -D-glucuronidase, lack the *ter* cluster, and are accordingly susceptible to tellurite and do not grow on CT-SMAC (Table 3); this is in agreement with the tellurite susceptibility of EHEC O165 strains isolated from patients in Japan (44). The SF phenotype of the U.S. EHEC O165:H25 strain was also reported for an EHEC strain of this serotype isolated in Finland (31).

In studies that associated human diseases with EHEC O165, the source of the infection remained unknown (17). However, in one of these studies, a fresh, homemade cheese consumed by the patient was suspected as the source of the EHEC O165 (17), suggesting that cattle are a possible reservoir. Indeed, isolation of EHEC O165:H25/NM from cattle feces and from beef has been reported (6, 24, 25, 26, 36). Some of these studies demonstrated that bovine EHEC O165:H25/NM strains share virulence genes, such as *eae*  $\epsilon$ , EHEC *hlyA*, and *efa1*, with human isolates of this serotype (6, 25). Moreover, as we show, such strains also share the *sfp* cluster, which encodes a putative adhesin (34). Taken together, these data suggest that cattle can harbor EHEC O165:H25/NM and can thus be sources of human infections.

EHEC O165:H25 bovine isolates can lose their large plasmids (and presumably the *sfp* cluster) during laboratory processing (25). We believe that such an event might explain why an EHEC O165:NM (*fliC*<sub>H25</sub>) strain we isolated from a patient in the 1990s (22) was *sfp* negative when it was screened several years later using *sfpA* PCR (23), thereby prompting us to consider that this locus was not found in strains belonging to the O165 serogroup (23). A recent more detailed analysis of this strain demonstrated that it lacks a large plasmid and all other genes typically located on pO165. This supports the hypothesis that this strain might have lost its *sfp*-harboring plasmid *in vivo* or during laboratory storage.

Most EHEC O157 and non-O157 strains possess large plasmids (37, 38, 39, 41, 46), which we also detected in EHEC O165:H25/NM. Analysis of colocalization of the *sfp* cluster with other plasmid genes (Table 1) and RFLP analysis of *sfp*-harboring plasmids demonstrated that the *sfp* cluster is located in EHEC O165 and O157 in several different plasmids, namely, (i) the 121-kb plasmid pSFO157 of strain 3072/96, harboring the *sfp* cluster together with EHEC *hlyA* and the *etp*

cluster but lacking *espP* and *katP* (Table 1); (ii) the ~80-kb plasmid pO165, which contains *sfp* in combination with EHEC *hlyA*, *espP*, and *katP* but lacks *etp* (Table 1) and structurally differs in strains from Germany and the United States (data not shown); and (iii) the ~120-kb plasmid pO165, harboring the *sfp* cluster together with EHEC *hlyA* and *katP* but without *espP* and *etp* (Table 1). The precise mechanisms of acquisition of these loci, including *sfp*, by EHEC strains are unknown. The megaplasmids (10, 11, 12, 13, 37, 38) are clearly mosaics, and most putative virulence genes are flanked by mobile genetic determinants, which probably facilitate their transfer. However, partial deletions of these mobile elements, as shown with the regions that flank the *sfp* operon in pO165, can stabilize particular virulence loci. Because EHEC O157:NM and O165:H25/NM are unrelated, we hypothesize that the *sfp* genes in these two serotypes were acquired independently in different events and from different sources. However, we cannot exclude the possibility of a direct transmission of the *sfp* cluster from one serotype to the other via horizontal transfer. It is interesting that although the *sfp* locus is present in EHEC O165:H25 strains associated with human disease in the United States, SF EHEC O157:NM, which regularly possesses this cluster, has not to date been isolated in the New World.

In SF EHEC O157:NM, the expression of Sfp fimbriae is strongly influenced by environmental conditions, in particular by oxygen tension (34). Studies are under way in our laboratory to determine conditions that promote Sfp expression in EHEC O165. Such studies will provide a closer insight into the role of Sfp fimbriae in the virulence of EHEC strains of these two serotypes.

In conclusion, the finding of a highly conserved *sfp* locus in divergent phylogenetic backgrounds prompts the need to perform further molecular and evolutionary comparative analyses of these unrelated EHEC lineages. It is interesting that suites of virulence genes seem to be common to human-pathogenic EHEC strains, where the possession of *stx* genes, for example, is almost always accompanied by the presence of one of the *eae* alleles (5, 9). Such associations occur much more frequently than would be expected by chance, and indeed, intimin and Stx interact in their effects on epithelial cells (42). To facilitate further studies on such convergence of virulence loci in phylogenetically diverse EHEC strains (33), the EHEC O165:H25 HUS isolate 820/08 characterized in this study is included in our recently created HUSEC reference collection (33; www.ehec.org).

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