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A Multiresistant Clone of Shiga Toxin-Producing *Escherichia coli* O118:[H16] Is Spread in Cattle and Humans over Different European Countries

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Multiresistant Shiga toxin-producing *Escherichia coli* (STEC) O118:H16 and O118 nonmotile strains (designated O118:[H16]) were detected by examination of 171 STEC isolates for their antimicrobial sensitivity. Of 48 STEC O118:[H16] strains, 98% were resistant to sulfonamide, 96% were resistant to streptomycin, 79% were resistant to kanamycin, 75% were resistant to tetracycline, 67% were resistant to ampicillin, 60% were resistant to chloramphenicol, 48% were resistant to trimethoprim, and 10% each were resistant to gentamicin and nalidixic acid. Nalidixic acid resistance and reduced susceptibility to ciprofloxacin were associated with the mutation *gyrA*$^{\text{LEU-83}}$. The STEC O118:[H16] strains were found to belong to a single genetic clone as investigated by multilocus enzyme electrophoresis and by multilocus sequence analysis of *E. coli* housekeeping genes. The STEC O118:[H16] strains originated from humans and cattle and were isolated in seven different countries of Europe between 1986 and 1999. Strains showing multiresistance to up to eight different antimicrobials predominated among the more recent STEC O118:[H16] strains. The genes in parentheses were associated with resistance to kanamycin (*aphA1-Ia*), chloramphenicol (*catA1*), tetracycline [*tet*(A)], and ampicillin ($bla_{\text{TEM-1}}$). Class 1 integrons containing *sulI* (sulfonamide resistance), *aadA1a* (streptomycin resistance), or *dfrA1* (trimethoprim resistance)-*aadA1a* gene cassettes were detected in 28 strains. The $bla_{\text{TEM-1b}}$ gene was present in 18 of 21 strains that were examined by nucleotide sequencing. Class 1 integrons and bla_{TEM} genes were localized on plasmids and/or on the chromosome in different STEC O118:[H16] strains. Hybridization of *Xba*I-digested chromosomal DNA separated by pulsed-field gel electrophoresis revealed that bla_{TEM} genes were integrated at different positions in the chromosome of STEC O118:[H16] strains that could have occurred by Tn*2* insertion. Our data suggest that strains belonging to the STEC O118:[H16] clonal group have a characteristic propensity for acquisition and maintenance of resistance determinants, thus contrasting to STEC belonging to other serotypes.

Escherichia coli O157 and certain other types of Shiga toxin-producing *E. coli* (STEC) are pathogenic for humans and can cause bloody diarrhea and hemolytic-uremic syndrome (HUS). Most of the human pathogenic STEC strains belong to a small number of O serogroups and carry the genes for production of Shiga toxins (*stx1* and/or *stx2*), for intimin (*eae*), and for enterohemorrhagic *E. coli* (EHEC) hemolysin (19).

In Germany, STEC belonging to O groups 157, 26, 103, 111, 118, and 145 were most frequently isolated from children with severe diarrhea and HUS (6). Some of these strains are also known as pathogens in calves (35, 36). Both STEC O118:H16 and O118:nonmotile (NM) (designated as STEC O118:[H16]) were most prevalent among STEC strains, which were isolated from diarrheic calves in Germany between 1986 and 1996 (35). STEC O118:[H16] strains from cattle and humans were found to be identical for their virulence markers and other traits, and two cases of transmission of STEC O118 from cattle to humans are described elsewhere (4, 34). The first cases of human infections with STEC O118:[H16] reported from Germany occurred in 1996 (6). Between 1996 and 1998, 23 patients infected with STEC O118 were identified in our laboratory. All cases were sporadic, most of the patients were children, and most of them were living in a rural environment (4).

By routine examination, we found that almost all STEC O118:[H16] strains from Germany were similar in the pattern of resistance to a number of antimicrobial drugs. We have therefore investigated 52 different motile and nonmotile STEC O118 strains from cattle and humans that were originally isolated between 1986 and 1999 in seven different European countries. Our results show that the STEC O118:[H16] strains belong to a distinct clonal group and that the isolates from different European countries are characterized by an increase of antimicrobial resistance that was observed over the the period of sampling.

MATERIALS AND METHODS

Bacteria. Forty-one STEC O118:H16 strains, seven STEC O118:NM strains, and four STEC O118:H12 strains were investigated, together with 119 STEC strains belonging to non-O118 serogroups. The sources and relevant properties of STEC isolates are described elsewhere (4, 6, 35). Of the 48 STEC O118:[H16] strains, 32 were from Germany, and the remaining 16 strains were obtained from

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Austria (Franz Allerberger, Universität Innsbruck), Belgium (Denis Piérard, Vrije Universiteit Brussel, and Jacques Mainil, Université de Liège), Denmark (Flemming Scheutz, Statens Seruminstitut, Copenhagen, Denmark), Italy (Alfredo Caprioli, Istituto Superiore di Sanità, Rome, Italy), Spain (Jorge Blanco, Universidad de Santiago de Compostela), and Switzerland (Louis Corboz, Universität Zürich).

Detection of STEC virulence markers. All strains were investigated for production of Shiga toxins (Stx) and for *stx*-specific DNA sequences, as well as for their hemolytic phenotypes and for the EHEC *hly* gene as described previously (4). Determination of the presence of the *eae* gene and genetic subtyping of *eae* variants were performed by PCR and restriction endonuclease digestion of PCR products as described previously (22).

Antimicrobial susceptibility tests. Antimicrobial susceptibility testing of bacteria was done by the disk diffusion method by using commercial disks (Oxoid, Wesel, Germany), according to the guidelines of the National Committee for Clinical Laboratory Standards (NCCLS) (20). Adjusted inocula of bacteria (ca. 5×10^4 CFU/ml) were inoculated on Mueller-Hinton agar (Oxoid) and incubated for 18 h at 35°C. Strains were considered resistant or sensitive by measuring the diameter of the growth inhibition zone, and interpretation of the results was done as recommended by the NCCLS (20). The antimicrobial agents tested, the loads of the disks, and sizes of growth inhibition of resistant strains, respectively, were as follows: sulfonamide (SUL; 300 μg, ≤12 mm), streptomycin (STR; 10 μg, ≤13 mm), kanamycin (KAN; 30 μg, ≤13 mm), tetracycline (TET; 30 μg, ≤14 mm), ampicillin (AMP; 10 μg, ≤13 mm), chloramphenicol (CHL; 30 μg, ≤12 mm), trimethoprim (TMP; 5 μg, ≤10 mm), gentamicin (GEN; 10 μg, ≤12 mm), and nalidixic acid (NAL; 30 μg, ≤13 mm). The STEC O118 strains were additionally investigated for the MICs of NAL and ciprofloxacin (CIP) by broth microdilution assay (21). The MIC was defined as the lowest concentration producing no visible growth. The breakpoints used to consider a strain as resistant were those recommended by the NCCLS (21) (NAL resistance, MIC ≥ 32 μg/ml; CIP resistance, MIC ≥ 4 μg/ml). In all tests, the susceptible strain *E. coli* ATCC 25922 was included for quality control.

Multilocus enzyme electrophoresis. To analyze enzyme electrophoretic variation, bacterial cell lysates were prepared and subjected to multilocus enzyme electrophoresis (28). Seventeen enzymes were examined for allelic variation as described previously (33). For each enzyme, electromorphs were determined through comparisions to standard mobility variants and assigned numbers by their rate of anodal migration. Isolates that lacked detectable enzyme activity were designated as a null allele at the locus in question. To estimate the genetic relationship among isolates, electromorphs were equated with alleles at the corresponding enzyme locus, so that each bacterial strain was characterized by its multilocus genotype (allele combination) for the enzyme-encoding loci assayed. Distinctive multilocus genotypes were designated electrophoretic types (ETs) and were numbered by their inferred relationships from phylogenetic analysis (33).

Multilocus sequencing methods. Oligonucleotide primers designed to amplify internal fragments for 13 housekeeping genes were used in multilocus sequence analysis (C. L. Tarr, T. M. Large, A. C. Bumbaugh, D. W. Lacher, and T. S. Whittam, unpublished data). Six of these genes were shown to be useful for identifying clonal frames in a previous study of pathogenic *E. coli* (24). The housekeeping genes include *arcA*, *aroE*, *aspC*, *clpX*, *cyaA*, *dnaG*, *fadD*, *grpE*, *icd*, *lysP*, *mdh*, *mtlD*, and *rpoS* (23). Amplification reactions consisted of 5 μl of 10× PCR buffer, 8 μl of deoxynucleoside triphosphate mix, 0.5 μl of each primer, 0.5 μl of Boehringer Mannheim *Taq* polymerase, and 34.5 μl of dH_2O (23). PCR was performed for 35 cycles under the following conditions: 1 min of denaturation at 92°C, 1 min of primer annealing at 57°C, and 15 s of extension at 72°C with an initial denaturing step of 94°C for 10 min. Amplicons were purified by using the Qiagen QiaQuick PCR purification kit. Cycle sequencing reactions (6 μl of CEQ mix, 1 μl of 20 μM primer and 3 μl of PCR product) were performed for 30 cycles under the following conditions: 96°C for 20 s, 50°C for 20 s, and 60°C for 4 min, with an initial denaturing step of 94°C for 1 min. Products were purified by using Sephadex columns and dried under vacuum centrifugation at room temperature. The samples were then rehydrated in 40 μl of formamide and sequenced by using a Beckman CEQ L automated sequencer. Data were analyzed by using the CEQ software and exported for analysis by using the SeqMan module of the DNAStar Lasergene software. Consensus sequences were aligned with CLUSTALX and the output files were modified for use in MEGA2 (14).

PCR for detection of antimicrobial resistance determinants. The detection of drug resistance genes (Table 1) was performed by PCR amplication and sequencing as described above. PCRs were performed with 5 μl of boiled bacterial cells (ca. 5×10^6 bacteria), or bacterial DNA (1 ng) in a 50-μl reaction mixture (1 U of Amplitaq Gold Polymerase; Perkin-Elmer Applied Biosystems, Weiterstadt, Germany; 5 μl of 10× buffer; 200 μM deoxynucleoside triphosphate; 1.25 mM $MgCl_2$; 5% dimethyl sulfoxide; 0.5 μM concentrations of each primer). DNA amplification was carried out in a GenAmp PCR system 9700 (PE Applied Biosystems, Foster City, Calif.). The PCR conditions were as follows: an initial hot start cycle at 94°C for 10 min, followed by 35 cycles of denaturation of 94°C for 1 min, primer annealing for 30 s at 55 to 65°C, and primer extension for 1 min at 72°C. A final incubation at 72°C for 10 min was performed after the last cycle. The PCR primers and the corresponding annealing temperatures are listed in Table 1. Detection of class 1 integron and gene cassettes located therein was performed according to conditions described elsewhere (11, 15). The specificity of the PCR products was determined by comparing the size of DNA fragments obtained by digestion with appropriate restriction endonucleases with the expected size deduced from the nucleotide sequence deposited in the GenBank (Table 1) and as described by Guerra et al. (11).

Nucleotide sequencing of the PCR products. The generated PCR products of bla_{TEM-1}, *gyrA*, and *parC* genes and class 1 integrons were purified with the QIAquick kit (Qiagen, Hilden, Germany). Sequencing reactions were carried out by using the Big Dye terminator cycle sequencing ready reaction kit (PE Applied Biosystems) according to the instructions given by the manufacturer. Automated nucleotide sequencing was performed with an ABI 377 Prism DNA Sequencer (PE Applied Biosystems) and documentation of sequence results was obtained by using the program AutoAssembler (PE Applied Biosystems). Sequences obtained were compared to those registered in the GenBank.

DNA hybridization analysis. Plasmid DNA was prepared as described previously (12). Conjugational transfer of resistance plasmids was performed as described by using the plasmid-free *E. coli* K-12 JC3272 as recipient (3). Plasmid DNA was separated on 0.7% agarose gels for Southern blotting. Preparation of samples for pulsed-field gel electrophoresis (PFGE) and Southern hybridizations of blotted PFGE gels were performed as described previously (5). The 828-bp PCR fragment generated by primers TEM-9F1/R1 (Table 1) from plasmid pBR322 was used as bla_{TEM}-specific DNA probe and the 1.6-kb fragment generated by primers 5′CS/3′CS (Table 1) from STEC O118:[H16] strain CB6525 was taken as the integron-specific DNA probe (15, 29; the present work). PCR probes were labeled with digoxigenin-11-dUTP (Roche Applied Science, Mannheim, Germany) as described and Southern hybridizations were conducted under conditions of high stringency (6).

RESULTS

A multiresistant clone of STEC O118:[H16] strains occurs in both humans and cattle. The investigation of STEC strains which were isolated from patients in Germany between 1996 and 1999 revealed that 19 of 26 (73.1%) of STEC O118:[H16] isolates were resistant to AMP, CHL, KAN, STR, SUL, and TET. In contrast, only a few of 119 examined non-O118 STEC strains that were isolated at the same time period showed resistance to one or more of these antibiotics, and the resistant strains were not associated with a particular serotype (Table 2). Human infections with multiresistant STEC O118:[H16] strains were observed over a period of several years. Because the cases were not epidemiologically linked (4), we became interested in the origin of the multiresistant STEC O118 strains. We have therefore investigated 41 STEC O118:H16, seven O118:NM, and four O118:H12 strains that were isolated in Austria ($n = 1$), Belgium ($n = 11$), Denmark ($n = 1$), Germany ($n = 35$), Italy ($n = 1$), Spain ($n = 2$), and Switzerland ($n = 1$) between 1986 and 1999.

By multilocus enzyme electrophoresis, the 52 STEC O118 strains were divided into two ETs (ET-A and ET-B), showing mobility differences in 7 of 17 employed enzymes. A total of 48 O118:[H16] strains from humans ($n = 28$) and cattle ($n = 20$) belonged to the ET-A clone. All ET-A strains were positive for stx_1 and for the intimin gene *eae*β1 (22). Three (6.25%) of the 48 ET-A strains carried an stx_2 gene and 44 (91.7%) were positive for plasmid-encoded EHEC hemolysin (EHEC-*hlyA*). Four strains belonging to the ET-B clone (O118:H12) were isolates from humans in Germany and were positive for the

TABLE 1. Primers used for the detection of integrons and resistance determinants

Target gene[a] (resistance)	Primer	Nucleotide sequence (5′-3′)	Reference	T_a (°C)[b]	Accession no.	Product size (bp)
Integron[c]	5′CS 3′CS	GGC ATC CAA GCA GCA AG AAG CAG ACT TGA CCT GA	15	55	AY007807	Variable
Integrase	intI1-F intI1-R	CCT CCC GCA CGA TGA TC TCC ACG CAT CGT CAG GC	4	58.5	AF071413	280
bla$_{TEM}$ (AMP)	TEM-9F TEM-9R	CAT TTC CGT GTC GCC CTT ATT CC GGC ACC TAT CTC AGC GAT CTG TCT A	This study	59	J01749	828
catA1 (CHL)	cat F1-F cat B2-R	AAG TTG GCA GCA TTC ACC CG TCG TGG TAT TCA CTC CAG AGC G	This study	61	J01841	573
tet(*A*) (TET)	tet(A)-F tet(A)-R	GGT CTT GCT CGT CTC GCT GG AAC GCC ATC CAT CCC CGT G	This study	62	X61367	690
aadA1 (STR)	aadA1a-F aadA1a-R	AAC GAC CTT TTG GAA ACT TCG G TTC GCT CAT CGC CAG CCC AG	This study	60	X12870	352
strA (STR)	strA-F strA-R	CCA ATC GCA GAT AGA AGG CAA G ATC AAC TGG CAG GAG GAA CAG G	This study	65	AJ249779	580
aphA1-Ia (KAN)	aph(3′)I-F aph(3′)I-R	AAC GTC TTG CTC GAG GCC GCG GGC AAG ATC CTG GTA TCG GTC TGC	25	65	J01839	670
aadB (GEN)	aadB-F[d] aadB-R[d]	GGG CGC GTC ATG GAG GAG TT TAT CGC GAC CTG AAA GCG GC	25	65	X04555	329
sulI (SUL)	sul1-F sul1-R	TGG TGA CGG TGT TCG GCA TTC GCG AAG GTT TCC GAG AAG GTG	17	62	U49101	790
QRDR *gyrA*	gyrA-P1 gyrA-P3	TGT CCG AGA TGG CCT GAA GC TGC CGT CAT AGT TAT CAA CGA	–[e]	58	U28377	374
QRDR *parC*	parC-3 parC-4	CCG TGC GTT GCC GTT TAT TG AAGTGCCGTCGAAGTTTGGCA	–[e]	58	U28377	368

[a] That is, it confers resistance to AMP, CHL, TET, STR, KAN, GEN, SUL, or it is the QRDR (quinolone resistance-determining region).
[b] T_a, annealing temperature.
[c] The *dfrA1* gene was identified by nucleotide sequencing and restriction enzyme digestion of PCR products generated with primers 5′CS and 3′CS.
[d] ant(2″)I-F and ant(2″)I-R (25).
[e] Unpublished data (B. Malorny, Federal Institute for Health Production of Consumer and Veterinary Medicine, Berlin, Germany).

stx$_{2d\text{-}OUNT}$ variant gene but negative for *eae* and EHEC-*hlyA* (4; data not shown).

To determine the genetic relatedness of the ET-A O118:[H16] clone to other pathogenic groups *E. coli* associated with diarrheal disease, we determined the nucleotide sequences for coding regions of 13 chromosomal loci encoding proteins with housekeeping functions. Comparison of the multilocus sequence data to the reference strain 93-111 (serotype O157:H7, clonal group EHEC 1), DEC 8b (O111:H8, EHEC 2), and B2F1 (O91:H21, STEC) indicated that the ET-A O118:[H16] strain CB5482 was most closely related to the EHEC 2 clonal group. The percentage of nucleotide difference in the total of 6,720 bp was 2.04 ± 0.18 (strain 93-111), 0.03 ± 0.02 (DEC 8b), and 0.40 ± 0.08 (B2F1). Comparison of the differences per 100 synonymous sites gave 6.65 ± 0.62 (strain 93-111, EHEC 1), 0.11 ± 0.08 (DEC 8b), and 1.35 ± 0.28 (B2F1). Thus, CB5482 has the lowest pairwise genetic distance to DEC 8b of the EHEC 2 group and is most distant from EHEC O157:H7. A phylogenetic analysis with these sequences shows that CB5482 belongs to the EHEC 2 group (Fig. 1), which also contains STEC strains with serotypes O26:H11 and O111:H8. Bootstrap confidence limits indicate that 100% of 1,000 randomly generated phylogenies place CB5482 in the EHEC 2 complex.

Among STEC O118 strains, resistance to the tested antimicrobials was only found in O118:[H16] ET-A strains (Table 2). Only one of the 48 STEC O118:[H16] ET-A strains and all four STEC O118:H12 ET-B strains were susceptible to all antimicrobials. STEC O118:[H16] ET-A strains showing resistance to

TABLE 2. Antimicrobial drug resistance in 48 STEC O118:[H16] ET-A and in 119 non-O118 STEC strains[a]

Antimicrobial drug	No. of resistant strains (%)	
	STEC O118:[H16] ET-A (n = 48)	STEC non-O118[a] (n = 119)
SUL	47 (97.9)	18 (15.1)
STR	46 (95.8)	18 (15.1)
KAN	38 (79.2)	3 (2.5)
TET	36 (75.0)	13 (10.9)
AMP	32 (66.7)	4 (3.4)
CHL	29 (60.4)	2 (1.7)
TMP	23 (47.9)	6 (5.0)
GEN	5 (10.4)	0 (<0.8)
NAL	5 (10.4)	0 (<0.8)

[a] Belonging to O serogroups O5 (n = 1), O18 (n = 1), O26 (n = 23), O76 (n = 3), O78 (n = 1), O84 (n = 2), O91 (n = 5), O103 (n = 6), O111 (n = 3), O113 (n = 3), O128 (n = 5), O157 (n = 33), O145 (n = 6), O146 (n = 6), O163 (n = 1), Orough (n = 18), and OX178 (n = 2).

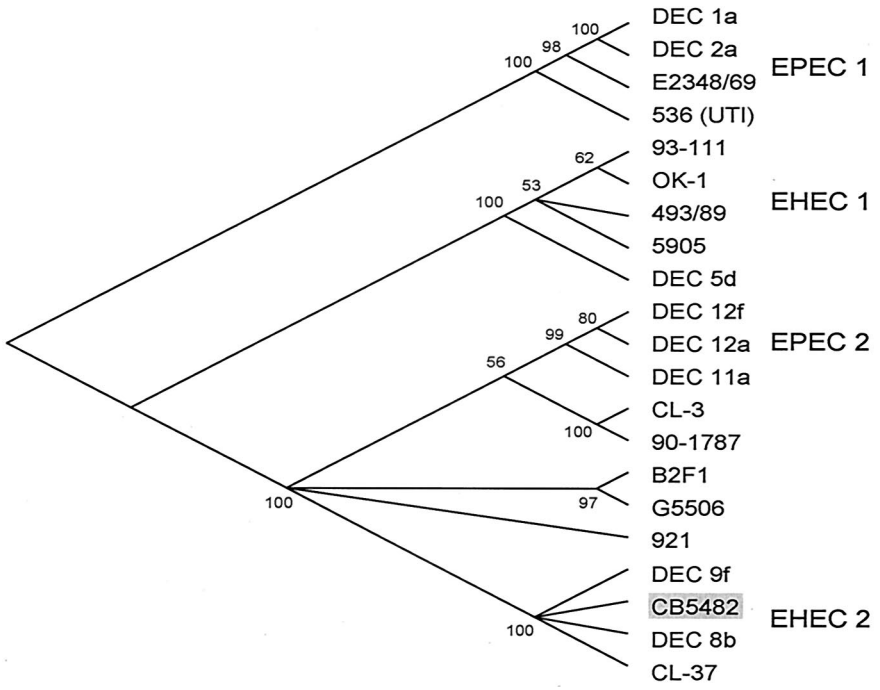

FIG. 1. Clonal phylogeny inferred from distances at synonymous sites (d_S) in gene sequences in 13 concatenated loci and inferred by the neighbor-joining algorithm. This is a consensus tree with the numbers at each node representing the percentage of boostrapped trees in which the node was observed. In addition to the O118:[H16] strain (CB5482), the pathogenic strains included in the figure are described by Reid et al. (23).

six or more of the drugs predominated among the isolates collected after 1994. The oldest isolates showing antibiotic resistance were from 1986 (STR and SUL), 1987 (KAN and TMP), 1988 (AMP), 1989 (CHL, GEN, and TET), and 1992 (NAL). Isolates from humans and cattle were not different from each other with regard to their resistance patterns and genes (Table 3).

Genes associated with antibiotic resistance in STEC O118:[H16] ET-A strains. The following genes were identified in STEC O118:[H16] ET-A strains resistant to the following: KAN (*aphA1-Ia*, 38 of 38 strains), CHL (*catA1*, 28 of 29 strains), TET [*tet*(*A*), 34 of 36 strains], and AMP (bla_{TEM}, 32 of 32 strains). A *strA* gene was found in 43 of the 46 STR-resistant strains and an *aadB* gene in two of the five strains that were resistant to GEN. A *sulI* gene was found in 29 of the 47 strains resistant to SUL. The three STR-resistant strains which were

TABLE 3. Antibiotic resistance phenotypes and genes in multidrug-resistant STEC O118:[H16] ET-A strains from humans and cattle in Europe

Resistance pattern[a] (no. of strains)	Resistance gene(s) (no. of strains carrying a given gene)	No. of isolates (country, yr of isolation)[b]	
		Human	Cattle
STR-SUL (5)	*strA*	1 (DK, 1999)	4 (B, 1986–1987)
STR-SUL-TET (1)	*aadA1a*, *sulI*, *tetA*	1 (A, 1997)	
STR-SUL-TMP-KAN (5)	*strA*, *dfrA1-aadA1a*, *sulI*, *aphA1-1a*		5 (B, 1987)
STR-SUL-KAN-TET-NAL (1)	*strA*, *aadA1a*, *sulI*, *aphA1-Ia*, *tetA*, $gyrA_{LEU-83}$	1 (I, 1992)	
SUL-TMP-TET-GEN (1)	*dfrA1-aadA1a*, *sulI*	1 (D, 1997)	
STR-SUL-TMP-KAN-TET-CHL (1)	*dfrA1-aadA1a*, *sulI*, *aphA1-Ia*, *tetA*, *catA1*	1 (D, 1997)	
STR-SUL-TET-CHL-GEN (1)	*strA*, *aadA1a*, *sulI*, *catA1*, *aadB*		1 (D, 1989)
STR-SUL-KAN-TET-AMP (4)	*strA*, *aphA1-Ia*, *tetA*, bla_{TEM-1}	3 (D, 1999)	1 (D, 1997)
STR-SUL-TMP-KAN-TET-AMP (1)	*strA*, *dfrA1-aadA1a*, *sulI*, *aphA1-IA*, *tetA*, bla_{TEM-1}		1 (CH, 1988)
STR-SUL-TMP-KAN-TET-AMP-CHL-GEN (3)	*strA*, *dfrA1-aadA1a*, *sulI*, *aphA1-Ia*, *tetA*, bla_{TEM-1}, *catA1*, *aadB* (1)		3 (D, 1994 and 1998)
STR-SUL-KAN-TET-AMP-CHL-GEN (1)	*strA*, *sulI*, *aphAI-Ia*, *tetA*, bla_{TEM-1}, *catA1*	1 (D, 1997)	
STR-SUL-TMP-AMP-CHL-NAL (1)	*strA*, *dfrA1-aadAla*, *sulI*, bla_{TEM-1}, $gyrA_{LEU-83}$	1 (D, 1996)	
STR-SUL-KAN-TET-AMP-CHL (11)[c]	*strA*, *aadA1a* (4), *sulI* (4), *aphA1-Ia*, *tetA*, bla_{TEM-1}, *catA1*, $gyrA_{LEU-83}$ (3)	8 (D, 1996–1999; B, 1991; SP, 1998)	3 (D, 1994–1998)
STR-SUL-TMP-KAN-TET-AMP-CHL (11)	*strA*, *dfrA1-aadA1a* (9), *sulI* (9), *aphA1-Ia*, *tetA*, bla_{TEM-1}, *catAI*	9 (D, 1996–1998; SP, 1996)	2 (D, 1994 and 1996)

[a] The relation gene and/or antimicrobial resistance is given in Table 1, except for *dfrA1*, which is implicated in TMP resistance.
[b] The country and year of isolation are indicated in parentheses: B, Belgium; D, Germany; I, Italy; CH, Switzerland; A, Austria; SP, Spain; DK, Denmark.
[c] Three strains were resistant to NAL and carried a $gyrA_{LEU-83}$ gene.

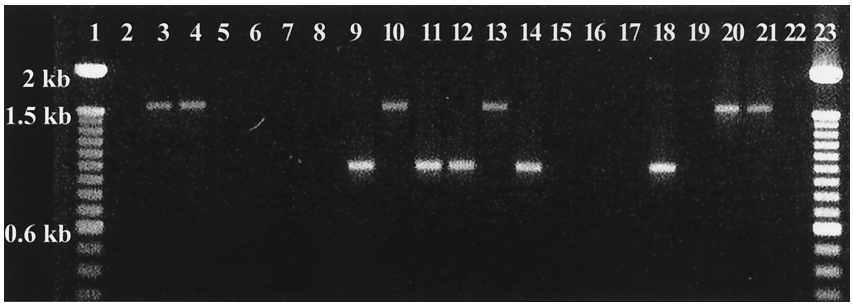

FIG. 2. Amplification of class 1 integrons from STEC O118:[H16] ET-A strains with primers 5′CS/3′CS (Table 1). Nineteen representative integron-positive and -negative strains from humans and cattle are shown. Lanes 1 and 23, 100-bp ladder; lane 2, CB7503 (negative); lane 3, CB7512 (*dfrA1-aadA1a*, 1.6 kb); lane 4, CB7882 (*dfrA1-aadA1a*, 1.6 kb); lane 5, CB8153 (negative with primers 5′CS/3′CS, positive for integrase and *sulI*); lane 6, CB8220 (negative); lane 7, CB8255 (negative); lane 8, CB8271 (negative); lane 9, E-D143 (*aadA1a*, 1.0 kb); lane 10, VTH28 (*dfrA1-aadA1a*, 1.6 kb); lane 11, VTH62 (*aadA1a*, 1.0 kb); lane 12, T17968 (*aadA1a*, 1.0 kb); lane 13, EC970130 (*dfrA1-aadA1a*, 1.6 kb); lane 14, 666/89 (*aadA1a*, 1.0 kb); lane 15, CB6069 (O118: H12, ET-B, negative); lane 16, 1874-99 (negative); lane 17, EH78 (negative); lane 18, RW2017 (*aadA1a*, 1.0 kb); lane 19, RW2030 (negative); lane 20, RW2173 (*dfrA1-aadA1a*, 1.6 kb); lane 21, RW2266 (*dfrA1-aadA1a*, 1.6 kb); lane 22, negative PCR control.

negative for the *strA* gene carried *aadA1a* genes, which are described as associated with integron structures (7).

We have therefore investigated the STEC O118:[H16] ET-A strains for the presence of class 1 integrons with the specific primers 5′CS/3′CS (15), and 28 of the 48 ET-A strains were found positive. Twenty-one strains generated amplification products of 1.6 kb that contained *dfrA1* and *aadA1a* gene cassettes (*dfrA1-aadA1a*), and the other seven strains generated PCR products of ca. 1.0 kb, which contained only the *aadA1a* gene cassette (Fig. 2). The 28 strains carrying the class 1 integron also carried the *sulI* gene. The other strain positive for *sulI* (CB8153) was positive for the integrase-specific PCR primers (intI1-F/R) but carried no *aadA1a* or *dfrA1* gene (Fig. 2).

Five strains showed resistance to NAL (MIC of >128 μg/ml) and reduced susceptibility to CIP (MICs of 0.5 and 0.25 μg/ml; one and four strains, respectively). Nucleotide sequence analysis of the quinolone resistance-determining region of the *gyrA* and *parC* genes of these strains showed a single mutation (Ser→Leu) in the codon 83 of the *gyrA* gene ($gyrA_{\text{LEU-83}}$).

STEC O118:[H16] ET-A strains are similar in their bla_{TEM} gene sequences. bla_{TEM} genes are found in a number of different genetic variants in *E. coli* and other *Enterobacteriaceae* (10). We used this polymorphism to investigate the relationships between AMP-resistant STEC O118:[H16] strains by nucleotide sequencing of an 828-bp stretch of the amplified bla_{TEM} gene in 21 STEC O118:[H16] ET-A strains that were isolated in Germany ($n = 17$), Belgium ($n = 1$), Switzerland ($n = 1$), and Spain ($n = 2$) (Table 4). Of the 21 STEC O118:[H16] strains, 18 were identical in that their bla_{TEM} genes all

TABLE 4. bla_{TEM} genotypes of 21 STEC O118:[H16] ET-A strains and location of bla_{TEM} genes[a]

Strain	Source (origin, isolation date [yr])	bla_{TEM} gene	bla_{TEM} gene location (size [kb])	Position in Fig. 3
EC970130	c (CH, 1988)	$bla_{\text{TEM-1b}}$	pl	
EH78	h (B, 1991)	$bla_{\text{TEM-1b}}$	pl	
VTH28	h (SP, 1996)	$bla_{\text{TEM-1b}}$	chr (ND)	
CB5482	h (D, 1996)	$bla_{\text{TEM-1b}}$	chr (459)	Lane 7, panels A and B
CB6175	h (D, 1996)	$bla_{\text{TEM-1b}}$	chr (459)	Lane 8, panels A and B; lane 9, panels C and D
CB7109	c (D, 1996)	$bla_{\text{TEM-1b}}$	chr (476)	Lane 8, panels C and D
CB6236	h (D, 1996)	$bla_{\text{TEM-1b}}$	chr (459)	Lane 6, panels A and B
CB6365	h (D, 1996)	$bla_{\text{TEM-1b}}$	pl and chr (331)	Lane 3, panels A and B; lane 1, panels C and D
CB6525	h (D, 1996)	$bla_{\text{TEM-1c}}$	pl and chr (105)	Lane 4, panels C and D
CB6585	h (D, 1996)	$bla_{\text{TEM-1b}}$	chr (459)	Lane 5, panels A and B; lane 5, panels C and D
CB6586	h (D, 1996)	$bla_{\text{TEM-1b}}$	chr (502)	Lane 4, panels A and B; lane 7, panels C and D
CB6981	c (D, 1996)	$bla_{\text{TEM-1b}}$	chr (502)	Lane 6, panels C and D
CB6980	c (D, 1997)	$bla_{\text{TEM-1b}}$	chr (ND)	
CB6888	h (D, 1997)	$bla_{\text{TEM-1b}}$	pl	
CB7014	h (D, 1997)	$bla_{\text{TEM-1b}}$	chr (476)	Lane 3, panels C and D
CB7035	h (D, 1997)	$bla_{\text{TEM-1b}}$	chr (47)	Lane 2, panels C and D
CB7099	h (D, 1997)	$bla_{\text{TEM-1a}}$	chr (ND)	
VTH62	h (SP, 1998)	$bla_{\text{TEM-1a}}$	chr (ND)	
CB7451	h (D, 1998)	$bla_{\text{TEM-1b}}$	chr (ND)	
CB7727	h (D, 1998)	$bla_{\text{TEM-1b}}$	chr (ND)	
CB7834	h (D, 1998)	$bla_{\text{TEM-1b}}$	chr (ND)	

[a] pl, plasmid > 93 kb; chr, chromosome; ND, not done; c, cattle; h, human. In parentheses are shown the sizes of the *Xba*I DNA fragments hybridizing with the bla_{TEM} gene probe.

showed mutations at positions 436 (C→T) and 604 (G→T). According to the updated *bla*$_{TEM}$ gene nomenclature the mutated genes are designated *bla*$_{TEM-1b}$ (10). One strain (CB6525) showed only a base substitution at position 436 (C→T) corresponding to the *bla*$_{TEM-1c}$ derivative and two other strains (CB7099 and VTH62) carried the original *bla*$_{TEM-1a}$ sequence (10) (Table 4).

Apart from the STEC O118:[H16] ET-A strains, only four STEC O26:H11 strains from our STEC collection were positive for *bla*$_{TEM}$ genes (Table 2). We examined the *bla*$_{TEM}$ genotypes of these strains by nucleotide sequence analysis. In contrast to the STEC O118 strains, all of these strains were found to be positive for the *bla*$_{TEM-1a}$ gene (data not shown), which suggests that *bla*$_{TEM-1a}$ has been acquired at least twice in the divergence of the EHEC 2 group.

Genetic location of class 1 integrons and *bla*$_{TEM}$ genes in STEC O118:[H16] strains. The genetic location of class 1 integrons was investigated in all 28 positive STEC O118:[H16] strains by Southern blot hybridization with a gene probe covering the *dfrA1-aadA1a* genes. Plasmid-encoded class 1 integrons were detected in 14 of the 28 strains. Further search for transferrable antimicrobial resistance revealed conjugative plasmids carrying *bla*$_{TEM}$ genes that encoded AMP resistance in the recipient strains. *bla*$_{TEM}$ genes harbored on large plasmids (>93 kb) were detected in 15 of the 32 AMP-resistant, *bla*$_{TEM}$-positive STEC O118:[H16] strains by DNA hybridization. Plasmids carrying both *bla*$_{TEM}$ genes and class 1 integrons were found in four strains (data not shown).

Of 32 *bla*$_{TEM}$-positive strains, 17 hybridized with the *bla*$_{TEM}$ probe only with chromosomal DNA. The similarity between strains with chromosomally inherited *bla*$_{TEM}$ genes was further investigated by determining the size of the hybridizing *Xba*I fragments from chromosomal DNA. *Xba*I fragments obtained from 11 strains were separated by PFGE, and the gels were blotted onto nylon membranes. With all 11 strains, only one *Xba*I fragment hybridized with the *bla*$_{TEM}$ gene probe. The probe hybridizing fragments were of different sizes: 502 kb (two strains), 476 kb (two strains), 459 kb (four strains), 331 kb (one strain), 105 kb (one strain), and 47 kb (one strain) (Fig. 3A and C and Table 4).

One pair of human (CB6586) and cattle (CB6981) strains that were isolated from a case of transmission between animals and humans (4) shared *bla*$_{TEM}$ probe hybridizing fragments of the same size (502 kb) (Table 4 and Fig. 3C and D). Another pair of STEC O118:[H16] strains, which were isolated on a farm from a calf and from a human, had identical *Xba*I profiles except for the *bla*$_{TEM}$ probe hybridizing fragment, which was 459 kb in the human (CB6175) and 476 kb in the calf isolate (CB7109) (Table 4 and Fig. 3C and D). The remaining seven STEC O118:[H16] strains were from single human cases and showed heterogeneous *Xba*I patterns that were to some extent associated with size differences in the *bla*$_{TEM}$ probe hybridizing fragments (Table 4 and Fig. 3).

DISCUSSION

STEC O118:[H16] strains have been identified as important agents of diarrhea in calves in Belgium and Germany (16, 35) and have been increasingly isolated from humans with diarrhea and HUS in Germany beginning in 1996 (4, 6). Human infections with STEC O118 are frequently associated with rural environments and multiple cases of transmission from cattle to humans have been described (4). These findings suggest that cattle are the major source of human infections with this organism.

By analysis of serotypes, virulence markers, and macrorestriction patterns of genomic DNA, STEC O118 were divided into three groups which were represented by three serotypes, O118:[H16], O118:H12, and O118:H30 (34). Multilocus enzyme electrophoresis revealed that STEC O118:[H16] and STEC O118:H12 strains represent two genetically different clonal types, ET-A and ET-B, which differ at 6 of 17 enzyme loci assayed.

The STEC O118:[H16] ET-A clone is a member of the EHEC 2 clone complex based on multilocus sequence analysis. Thus, the clone marked by ET-A is most closely related to STEC clones with serotypes O26:H11 and O111:H8 (33). The fact that all STEC O118:[H16] ET-A strains were positive for intimin β is consistent with other members (O26:H11 and RDEC) of the EHEC 2 group.

Resistance to two or more classes of antimicrobials was found as a characteristic trait in 47 (97.9%) of 48 STEC O118:[H16] ET-A strains that were collected from humans and cattle in Germany and in some other European countries between 1996 and 1999. In contrast, antimicrobial resistance was less frequently (15.1%) expressed by STEC belonging to other serotypes that were isolated at the same time period, and the resistant strains were not associated with a particular serotype. Similar findings were made in studies performed in the United States (13.3% of STEC were resistant) and Japan (8.7% of STEC were resistant) (13, 27).

The origin of the strains and the differences found in their *Xba*I PFGE patterns indicate that most of STEC O118:[H16] ET-A strains from our study were epidemiologically unrelated to each other; however, similar antimicrobial resistance patterns were found in strains of different origin and source (4; this work). Analysis of genes encoding resistance to KAN, CHL, TET, and AMP revealed a remarkable homology between STEC O118:[H16] strains showing resistance to these compounds. This finding could indicate that the resistant strains are clonally related, having originated once from a common ancestor with these resistance determinants. An alternative hypothesis is that resistance has evolved multiple times in the STEC O118:[H16] ET-A clone because members of the clone have a particular propensity for acquiring and incorporating resistance (R) genes. R genes could have been aquired by uptake of R plasmids, which were found to be present in many of the STEC O118:[H16] ET-A strains. In a further step, the R genes could have been incorporated into the chromosome of the bacteria as was found to be the case with many strains for class 1 integron-associated R genes (*dfrA1*, *aadA1a*, and *sulI*) and for the *bla*$_{TEM}$ genes. A chromosomal location of *bla*$_{TEM}$ genes was only rarely described in strains of *E. coli* (31) and is therefore a remarkable feature of STEC O118:[H16] ET-A strains. *bla*$_{TEM}$ genes were reported to be associated with different transposons, including Tn*2* (10). By using a PCR with one primer specific for *tnpR* of Tn*2* in combination with a *bla*$_{TEM-1}$-specific primer we have found that transposon-specific sequences were closely linked to chromosomally and plasmid-encoded *bla*$_{TEM-1}$ genes in 12 of the

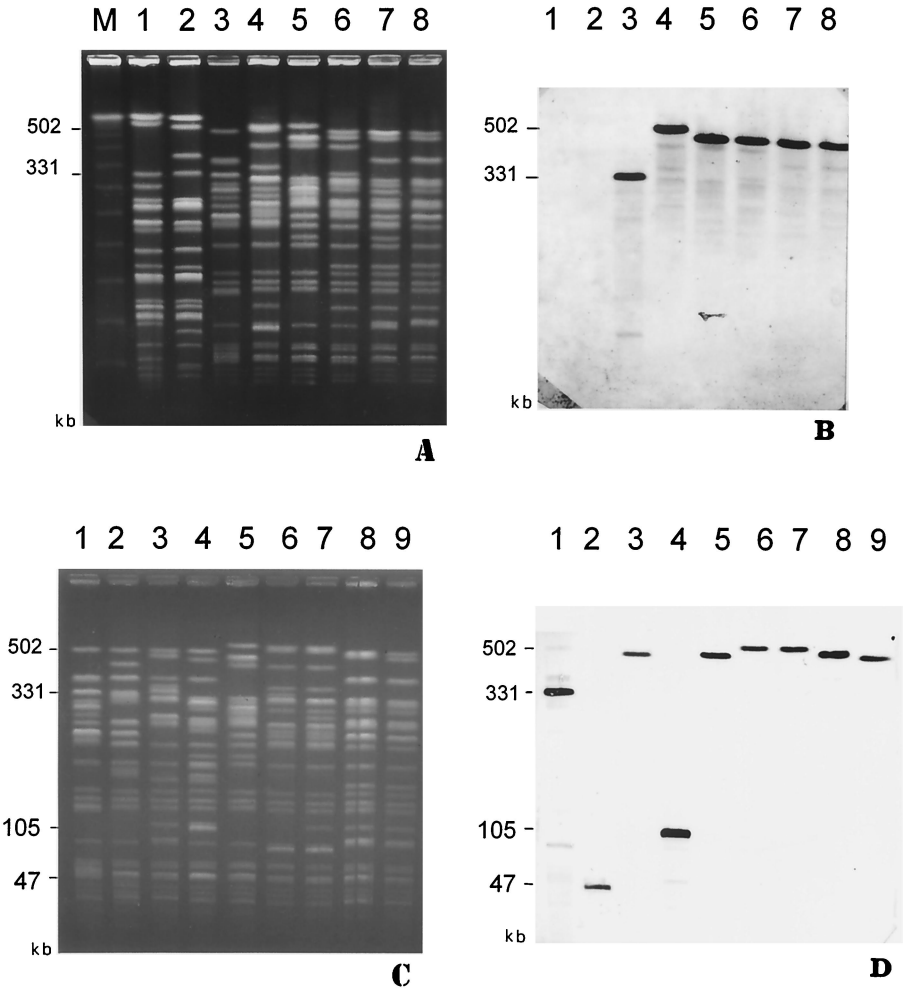

FIG. 3. (A) PFGE analysis of *Xba*I-digested total DNA of STEC O118:[H16] ET-A strains. Lanes: M, molecular weight standard (lambda concatemers; Bio-Rad Laboratories); 1, CB6591 (O118:H12, bla_{TEM}-negative); 2, CB6069 (O118:H12, bla_{TEM} negative); 3, CB6365 (O118:H16, $bla_{TEM\text{-}1b}$); 4, CB6586 (O118:NM, $bla_{TEM\text{-}1b}$); 5, CB6585 (O118:H16, $bla_{TEM\text{-}1b}$); 6, CB6236 (O118:H16, $bla_{TEM\text{-}1b}$); 7, CB5482 (O118:H16, $bla_{TEM\text{-}1b}$); 8, CB6175 (O118:H16, $bla_{TEM\text{-}1b}$). The positions of some *Xba*I fragments are indicated in kilobases at the left side of the gel. (B) Hybridization of DNA from strains shown in panel A with the bla_{TEM}-specific gene probe. (C) PFGE analysis of *Xba*I-digested total DNA of STEC O118:[H16] ET-A strains. Lanes: 1, CB6365 (O118:H16, $bla_{TEM\text{-}1b}$); 2, CB7035 (O118:H16, $bla_{TEM\text{-}1b}$); 3, CB7014 (O118:H16, $bla_{TEM\text{-}1b}$); 4, CB6525 (O118:H16, $bla_{TEM\text{-}1c}$); 5, CB6585 (O118:H16, $bla_{TEM\text{-}1b}$); 6, CB6981 (O118:NM, $bla_{TEM\text{-}1b}$); 7, CB6586 (O118:H16, $bla_{TEM\text{-}1b}$); 8, CB7109 (O118:H16, $bla_{TEM\text{-}1b}$); 9, CB6175 (O118:H16, $bla_{TEM\text{-}1b}$). The positions of some *Xba*I fragments are indicated in kilobases at the left side of the gel. (D) Hybridization of DNA from strains shown in panel A with the bla_{TEM}-specific gene probe.

STEC O118:[H16] ET-A strains (data not shown). It was reported that Tn*2* can insert at multiple sites in the bacterial chromosome (30) and the heterogeneous hybridization patterns that were obtained with the bla_{TEM} gene probe and *Xba*I-digested genomic DNA of STEC O118:[H16] ET-A strains indicate that the bla_{TEM} genes are integrated at multiple sites in the chromosome of these strains.

The finding that strains showing resistance to eight different antimicrobial agents predominated among the most recently isolated STEC O118:[H16] strains supports the hypothesis that STEC O118:[H16] ET-A strains have a tendency to acquire and accumulate R determinants in nature. The presence of resistance to NAL and reduced susceptibility to CIP in 9.4% of the strains is a cause of concern. Additional amino acid changes within the quinolone resistance-determining region of the gyrase (*gyrA* and *gyrB*) and the topoisomerase IV (*parC*) genes could give rise to fluoroquinolone resistance (18, 32). In *E. coli* this resistance is generally associated with mutations affecting the amino codon Ser83 or Asp87 of *gyrA* in a first step and with Ser80 of *parC* in a secondary step (18, 32). The five STEC O118:[H16] ET-A strains of our series presented a single point mutation in *gyrA*, which conferred the reduced susceptibility to ciprofloxacin, and thus we could consider these strains as progenitors for the evolution of full resistance.

Resistance properties can provide a selective advantage if the bacteria are frequently exposed to antimicrobial substances, which is often the case in livestock on farms. Indeed, multiresistant STEC O118:[H16] strains were isolated from cattle housed at different farms in Belgium, Germany, and Switzerland. For more than four decades, it has been a common practice on farms to use antimicrobial agents for disease prevention and growth promotion of animals. The widespread

use of antimicrobial agents would select for resistance and may have promoted the increasing frequency of STEC O118:[H16] ET-A strains in the bovines (16, 35). As a consequence, humans became more likely to be exposed to these organisms via the food chain and by direct and indirect transmission from cattle, and this could explain why infections with this pathogen have increased particularly in the rural population (4). It has been shown that STEC can survive for long time periods in fecally contaminated soil and water (1, 8, 9). Further dissemination of STEC O118 to farmland and to noninfected cattle could have occurred by irrigation of soils with contaminated surface water and by direct and indirect transmission of bacteria to noninfected animals.

Our findings show that certain types of STEC, such as STEC O118:[H16] ET-A strains, are well adapted to survive under antibiotic pressure. A study performed at the University Hospital in Dusseldorf, Dusseldorf, Germany, has revealed that class 1 integron-carrying *E. coli* and other *Enterobacteriaceae* have increased from 4.7 to 17.4% between 1993 and 1999 (26). Furthermore, our own investigations show that 12.5% of a series of 300 German *E. coli* strains, belonging to different serotypes, isolated between 1999 and 2001 from animal sources (cattle, avian, and swine), carried class 1 integrons. At least five different gene cassette arrays were found within these integrons. Among them, *dfrA1-aadA1a* and *aadA1a* were the most frequently encountered (unpublished data). Integron-associated antimicrobial resistance was also found in other STEC belonging to different serotypes, including O157:H7, which were isolated from humans, animals, and food in the United States (37). Although antimicrobial therapy is generally not recommended for treatment of STEC infections in humans, the indirect selection for multiresistant strains will contribute to the increase of emerging, antimicrobial-resistant pathogens, such as STEC O118:[H16], and facilitate the spread of these mobile resistance elements to other bacteria. An increased surveillance and the development of adequate prevention strategies are needed for public health reasons.

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