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SPONTANEOUS ANTIBIOTIC RESISTANCE MUTATION ASSOCIATED PLEIOTROPIC CHANGES IN *ESCHERICHIA COLI* O157:H7

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Besides the well-known O157:H7 clone causing enterohaemorrhagic colitis and haemolytic uraemic syndrome in Europe, Japan and North America, the number of *Escherichia coli* isolates with non-motile (NM) phenotype has considerably increased. We supposed that spontaneous antibiotic resistance mutation could cause this phenotypic change. To model our hypothesis we isolated rifampicin- (Rif) and ampicillin- (Amp) resistant mutants from *E. coli* O157:H7 prototype strains 7785 and EDL933. Among Rif^r mutants we could isolate strains with no or reduced motility, while the Amp^r mutants became hypermotile. The biochemical profile of the mutants had not changed but phage sensitivity and generation time of the mutants were altered. Among the representative strains we did not find polymorphism with Southern blot analysis and no polymorphism was found in the *fliC* gene of the mutants. The described characteristics have proven to be stable. In a mice virulence assay by intravenous infections the virulence of the derivatives was also found to be changed. In summary, we found that the antibiotic-resistant phenotype in *E. coli* O157:H7 was coexpressed with several other phenotypic changes including motility and virulence. It can be assumed that expression of the involved phenotypes may be under the influence of a common regulatory cascade. Further work is needed to identify the components and mechanism of this regulatory system.

Key words: *Escherichia coli*, O157:H7, non-motile, antibiotic resistance, mutation, flagella, *fliC*

Escherichia coli O157:H7 was first recognised as a human pathogen in 1982 (Riley et al., 1983). It has been found to be the primary cause of haemorrhagic colitis (HC) and the most common cause of haemolytic uraemic syndrome (HUS). Severe food-borne outbreaks have been reported worldwide (Griffin and Tauxe, 1991). Clonal analysis of enterohaemorrhagic *E. coli* O157 (EHEC) strains associated with HC and HUS has demonstrated that they represent two

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divergent clones. According to a model the two branches have evolved from an O55:H7 ancestor (Feng et al., 1998). One branch has lost several metabolic functions (e.g. sorbitol fermentation) and resulted an O157:H7 clone that has spread worldwide. The other branch retained the ability to ferment sorbitol, but has lost its motility, and emerged mostly in Central Europe (Gunzer et al., 1992; Karch et al., 1993; Bielaszewska et al., 1998; Liesegang et al., 2000; for review, see Caprioli and Tozzi, 1998; Karch and Bielaszewska, 2001). In addition, the frequency of sorbitol-non-fermenting O157:NM (NM indicates non-motile) isolates has also increased dramatically in the last few years in the USA. The previously rare *E. coli* O157:NM isolates increased from 6% of the total Shiga toxin (Stx) producing isolates in the early 1990s to 47% in 1996 (Fields et al., 1997). It was also revealed by PCR-restriction fragment length polymorphism (PCR-RFLP) that the entire coding sequence of the *fliC* gene, encoding the single structural flagellin subunit, restricted with *Rsa*I gives an identical pattern for *E. coli* O157:H7 and O157:NM strains (Fields et al., 1997). Another study determining and comparing the entire *fliC* gene of *E. coli* O157:H7 and O157:NM strains revealed an early stop codon in the *fliC* gene leading to truncated protein in the non-motile strain (Reid et al., 1999).

The aim of this study was to examine the possible molecular basis of H7 antigenic variation in enterohaemorrhagic *E. coli* O157 to determine whether spontaneous mutation in the *fliC* gene could be the only possible cause of the loss of motility and change of serotype of *E. coli* O157 strains as reported by Reid et al. (1999) or whether the H7 antigenic change may be determined by other factors as well. In *E. coli* rifampicin resistance (Rif^r) is associated with specific nucleic acid substitutions in the gene encoding for RNA polymerase subunit β (*rpoB*) described by Jin and Gross (1988) and it has been found that rifampicin-resistant *rpoB* mutants cause pleiotropic phenotypes including growth phenotypes, ability to support different bacteriophages, ability to maintain the F' episome, interaction with mutant alleles at other loci, sensitivity to uracil, inhibition by 5-fluorouridine, and dominance recorded by Jin and Gross (1989). On the basis of these observations rifampicin- and ampicillin-resistant mutants were isolated and the motility, the production of FliC protein and flagella and the virulence of these antibiotic-resistant derivative strains were compared. The present study revealed that the Rif^r *E. coli* O157 mutant strains became less or non-motile and showed pleiotropic phenotypic changes. Interestingly the ampicillin-resistant mutants also showed pleiotropic alterations including a novel phenotype: these mutants became hypermotile and produced increased amounts of FliC protein and flagella.

Materials and methods

Bacterial strains. The *E. coli* strains used in this study and their description are shown in Table 1. C57 and C81 were kindly provided by Hedda Milch, 'Johan Béla' National Epidemiological Center, Budapest, Hungary.

Table 1

Escherichia coli strains studied

Strain	Description	Reference
7785	First human *E. coli* O157:H7 haemorrhagic colitis case isolate	Riley et al., 1983 Wells et al., 1983
7785-5	Rifampicin-resistant derivative of 7785	Tóth et al., 1990
7785-1-4 and 7785-6-118	One hundred seventeen rifampicin-resistant derivatives of 7785	This study
7785-A1-3	Three ampicillin-resistant derivatives of 7785	This study
EDL933	*E. coli* O157:H7 hamburger isolate, implicated as the vehicle of the first HC outbreak. Full genome is sequenced	O'Brien et al., 1983 Perna et al., 2001
EDL933-1-10	Ten rifampicin-resistant derivatives of EDL933	This study
C57	*E. coli* O157:H7 strain isolated from human patient in Hungary	This study
C81	*E. coli* O157:NM strain isolated from human patient in Hungary	This study
C600	Streptomycin-resistant mutant of *E. coli* K-12	Bachman, 1996
J 53	Rifampicin-resistant mutant of *E. coli* K-12	Bachman, 1996

Selection of antibiotic-resistant mutants of E. coli *O157:H7.* Overnight Luria broth (LB) culture of *E. coli* O157:H7 strains 7785 and EDL933 was plated on nutrient agar plates containing antibiotic. For selection 100 mg/l rifampicin (Rifamycin SV sodium salt, Pharmachim, Bulgaria) and 12.5 mg/l ampicillin (KRKA, Novomesito) were used.

Antibiotic resistance. Antibiotic resistance of the wild-type strains and mutants was examined by disk diffusion method using the following antibiotics: streptomycin, spectinomycin, chloramphenicol, tetracycline, neomycin, ampicillin, kanamycin, gentamicin, nalidixic acid, nitrofurantoin and rifampicin.

Motility assay. The motility of bacterial strains was examined either in U tubes containing 0.5% semisolid LB agar with and without anti-H7 serum (immobilisation) or on Petri dishes as described earlier (Farmer and Davis, 1985; Allison et al., 1994) and in tubes containing Hitchens medium. In these experiments the cultures were incubated for 18–24 h at 37 °C.

Agglutination. H7 titre of parent *E. coli* O157:H7 strains and antibiotic-resistant mutants was determined by standard tube agglutination assay using two-fold dilutions of anti-H7 immune serum.

Phages. Phage sensitivity of parent *E. coli* O157 and antibiotic-resistant mutant strains was tested with *E. coli* (Milch and Gyenes, 1972) and *Shigella sonnei* (Hammarström, 1949).

Growth rates. Strains were grown for 18 h in LB at 37 °C. Cultures were diluted in fresh LB about 200 times to the same cell density at 600 nm wavelength and grown at 37 °C with shaking at 200 rpm. OD_{600} measurements were taken every 30 min.

Biochemical profiles. The 32 main biochemical characteristics were determined by API identification (Bio Merieux) program (rapid ID32E), and sorbitol fermentation was tested on MacConkey agar plates containing 1% sorbitol.

SDS-PAGE. Secreted bacterial proteins were precipitated with trichloroacetic acid (TCA) as described by Brunder et al. (1997) and samples were separated on 10% SDS-polyacrylamide gels as described by Laemmli (1970). Gels were stained with 0.2% Coomassie brillant blue R250 (CBB) and destained by standard technique or the proteins were electrotransferred onto nitrocellulose membranes for immunological detection or to 0.2 μm PVDF membrane for N-terminal sequencing.

Amino acid sequencing. The exoproteins were separated by SDS-PAGE and blotted to PVDF membrane. Blotted proteins were visualised with 0.2% CBB in 50% methanol, and destained with 50% methanol and 10% acetic acid. The band of interest was excised and the N-terminal was sequenced in the Protein and Nucleic Acid Sequencing Laboratory of the Agricultural Biotechnology Center, Gödöllő by Applied Biosystem sequencer.

Immunoblot analysis. Antiserum was produced in rabbit by intravenous injections of formalinised suspension of strain 7785 in Penassay broth (Antibiotic medium 3) and the resulting antiserum was absorbed intensively with its Rif^r mutant strain, as described earlier (Tóth et al., 1990). This absorbed immune serum and the H7 immune serum (kindly provided by Éva Czirók, 'Johan Béla' National Epidemiological Center, Budapest, Hungary) were used in 1:400 dilutions. As a blocking and diluting agent 5% skimmed milk protein and 0.3% Tween 20 in 10 mM Tris pH 7.5 and 0.9% NaCl were used. Anti-rabbit IgG alkaline phosphatase conjugate (BioRad) was used as second antibody in 1:2000 dilution.

Electron microscopy. Bacterial cultures were grown with and without shaking in LB at 37 °C overnight. Cultures were washed twice with sterile phosphate buffered saline (PBS), and one drop of these suspensions was placed on grids covered with celloidine, stained with 1% phosphotungstic acid (pH 7) and examined by transmission electron microscope (Jeol 100C).

Immunoelectron microscopy. Absorbed immune serum was used in 1:50 dilution and a protein A-gold conjugate was used as secondary antibody also in 1:50 dilution.

In vivo *virulence test.* The intravenous mouse lethality test was performed as described earlier (Morschhäuser et al., 1994; Fuchs et al., 1999). Briefly, bacteria were grown overnight in LB at 37 °C. The bacterial cells were washed twice with sterile PBS and photometrically (Spekol, Germany) reconstituted to the desired cell density at 690 nm wavelength. Female specific pathogen free (SPF) CFLP mice (Gödöllő, Hungary) of 20 g body weight were infected intravenously with 2.5×10^8 colony forming units (CFU) of bacteria in 0.5 ml PBS. In three independent experiments a total of 50 mice were infected with all studied strains. As negative control, the mice were infected with the same CFU of an apathogenic *E. coli* K-12 strain. Animals were observed for 14 days and differences between the strains in terms of death were calculated by using the chi-squared test.

DNA techniques

Genomic DNA isolation, DNA cleavage with restriction enzymes and agarose gel electrophoresis were performed according to standard procedures (Sambrook et al., 1989).

PCR. To amplify the K-12 specific *fliC* gene product (accession number: AE000285) the following PCR primers were used: 5'-GTTAGCTTTTGCCAA CACGGAG-3' (position 1750-1771) and 5'-AAACTGCAGGATTCCAAGGGT-3' (position 2272-2293). The entire *fliC* gene was amplified from parent O157:H7 and antibiotic-resistant derivative strains by using F-FLIC1 and R-FLIC2 primers as described by Fields et al. (1997). The presence of *stx1*, *stx2* and *eae* genes was tested as described by China et al. (1996).

Southern hybridisation. 10 μg of DNA samples were digested with *Bam*HI and *Hin*fI and separated in 0.8% gel. Separated DNA fragments were transferred to positively charged nylon membrane (Amersham Pharmacia Biotech Ltd.) by vacuum blotting as recommended by the supplier (BioRad), and were probed with ^{32}P labelled K-12 specific *fliC* amplicon.

DNA sequencing. The PCR-amplified entire *fliC* gene was purified by a PCR purification kit (Qiagen). Nucleotide sequence reactions were set up with a dye terminator cycle sequencing ready reaction kit, and the sequences were determined with an ABI Prism 377 DNA sequencing system.

Results

Phenotypic characters of Rif- and Amp-resistant E. coli *O157:H7 mutants.* Using rifampicin as selective agent we isolated Rif^r mutants from prototype *E. coli* O157:H7 strains 7785 and EDL933 with a mutation frequency of approxi-

mately 2×10^{-8}. For motility assays 117 Rif^r 7785 and 10 Rif^r EDL933 mutants were randomly selected and we also included an earlier described rifampicin-resistant mutant strain 7785-5 (Tóth et al., 1990). By using ampicillin selection three ampicillin-resistant mutants were isolated from 7785 and were also investigated further.

Motility. The motility of the 128 Rif^r and 3 Amp^r mutants was compared with the parent O157:H7 strains in LB soft agar. The motility of freshly isolated human pathogenic strain C57 of O157:H7 and C81 of O157:H$^-$ was also investigated. After incubation for 16–24 h some Rif^r mutants failed to migrate through the Hitchens agar or showed reduced motility in contrast to wild-type strains 7785, EDL933 and C81. Out of the 118 Rif^r mutant 7785 strains 3 became non-motile and 14 became less motile. In contrast, all the three Amp^r 7785 mutants migrated faster than parent 7785. The motility of parent and three antibiotic-resistant derivative strains, representing the different motility phenotypes, in semisolid agar is shown in Fig. 1. Amp^r 7785-A2 was hypermotile, while Rif^r 7785-5 had reduced motility and 7785-15 was non-motile. One Rif^r mutant of EDL933 showed non-motile phenotype.

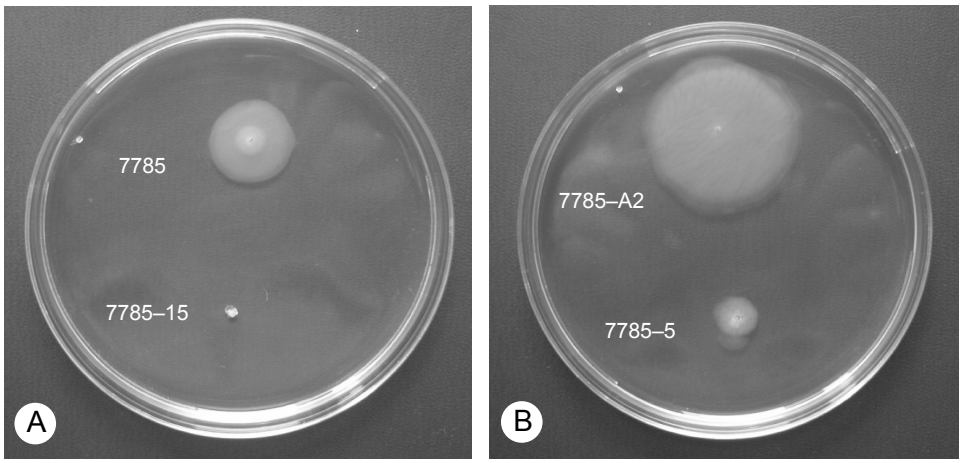

Fig. 1. Motility of parent 7785, Amp^r 7785-A2, Rif^r 7785-5 and Rif^r 7785-15 was compared in LB agar plates containing 0.5% agar. The plates were incubated at 37 °C for 8 hours. The Amp^r derivative migrated faster than parent 7785 and the Rif^r derivatives had reduced motility

To prove that parent 7785 and EDL933 strains do not contain non-motile derivatives, the motility of 10 randomly selected colonies of each parent strain was examined. No reduction in the motility was detected in U tubes. H7 immune serum immobilised all of the *E. coli* O157:H7 and the motile Rif^r mutants.

The H7 titres of the parent strains and the corresponding antibiotic-resistant mutant strains were determined by tube agglutination. High H7 titre was measured in parent strains: 1:1600 (7785) and 1:800 (EDL933), respectively. One representative Rif^r mutant, 7785-5 with reduced motility had four times less H7 titre than the corresponding parent strain, and the non-motile Rif^r derivatives 7785-15 had no detectable H7 titres. Amp^r mutants kept the original H7 titre.

Antibiotic resistance. The two prototype strains of O157:H7 and the freshly isolated pathogenic strains C75 and C81 were sensitive to streptomycin, spectinomycin, chloramphenicol, tetracycline, neomycin, ampicillin, kanamycin, gentamicin, nalidixic acid, nitrofurantoin and rifampicin. None of the Rif^r and Amp^r mutants of 7785 became resistant to further antibiotics.

Electron microscopy and immunoelectron microscopy. Electron microscopy studies of parent 7785 and corresponding Rif^r and Amp^r resistant mutants demonstrated that reduction, lack and alteration of motility observed in these strains were accompanied by changes in flagella expression. Strain 7785 showed peritrichous arrangement of flagella (Fig. 2A), while 7785-5 produced less flagella (Fig. 2B). No flagella were observed on the surface of the Rif^r NM 7785-15 derivative (Fig. 2C). The hypermotile Amp^r 7785-A2 derivative produced at least as many flagella as the parent 7785 did, and cells showed branched flagella (Fig. 2D), as demonstrated by transmission electron microscopy.

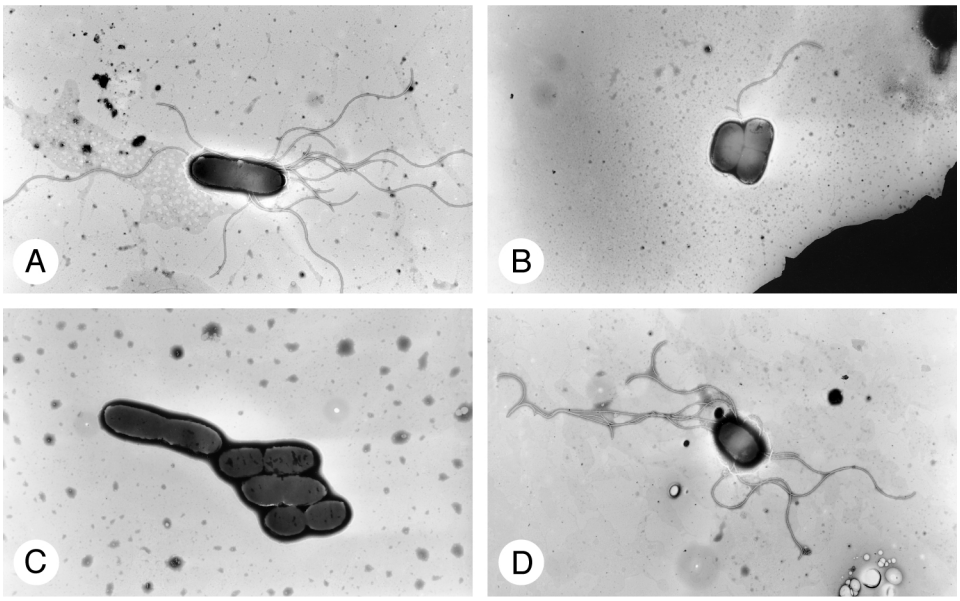

Fig. 2. Electron micrographs of parent 7785 (A), less motile 7785-5 (B), non-motile 7785-15 (C) and hypermotile 7785-A2 (D) derivative strains. Magnification in each panel was × 2,700

The antibodies, raised against parent 7785 and absorbed with Rif^r NM mutant specifically bound to flagella, demonstrated by immunoelectron microscopy (Fig. 3). On the other hand, the same absorbed antibody did not bind to the surface of the NM Rif^r mutant (data not shown).

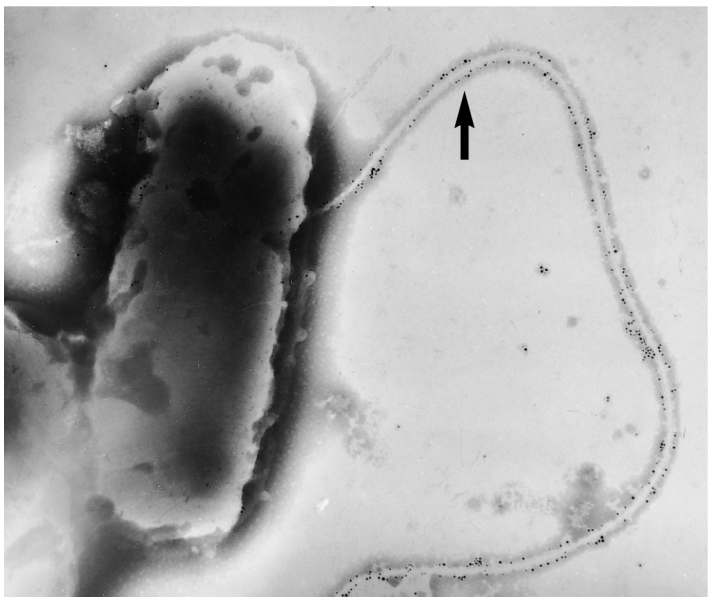

Fig. 3. Immunoelectron micrograph of 7785. The colloid gold labelled absorbed immune serum specifically bound to the flagella (indicated by arrow). Magnification was × 32,400

Protein analysis

To find whether the Rif^r and Amp^r mutants synthesised different amounts of FliC protein, exoprotein fractions of parent O157:H7 and the corresponding antibiotic-resistant mutants were compared by SDS-PAGE and by immunoblot analysis.

SDS-PAGE. A typical SDS-PAGE of exoprotein fractions of the representative strains is shown in Fig. 4A. TCA-precipitated exoprotein fractions of the wild-type 7785 (lane 2), the corresponding Rif^r mutants (lanes 3–5), an O157:NM (C81; lane 6) and an O157:H7 (C57, lane 7) wild-type strains were separated by a 10% SDS-PAGE and stained with CBB. The exoprotein fraction of O157:H7 strains contained a 60-kDa protein, indicated by an arrow, which was missing from the Rif^r 7785 mutants and from the O157:NM wild-type strain.

N-terminal sequence. The N-terminal sequence of the 60-kDa exoprotein was determined. The repeatedly obtained AQVIN amino acid sequence corresponds to only one protein and data search revealed 100% identity with the first 2–6 residues of *fliC* flagellin of *E. coli* (M_r: 60.5 kDa).

Immunoblot analysis. On the basis of SDS-PAGE and immunoelectron microscopy studies immunoblot analysis was carried out with selected strains. In these experiments the absorbed immune serum (Fig. 4B) and the H7 diagnostic immune serum (data not shown) bound to the FliC flagella protein of 7785 (lane 2), C57 (lane 4), EDL933 (lane 6), but no FliC could be detected in any of the non-motile Rif^r 7785-15 (lane 3) or in C81 (lane 5).

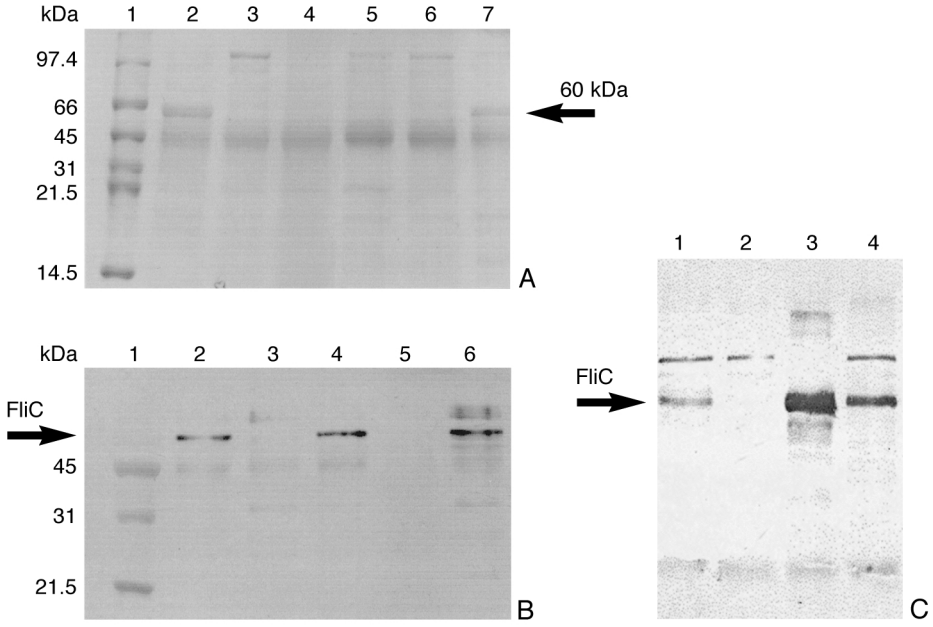

Fig. 4. Protein analysis. Coomassie blue stained SDS-PAGE (A) and Western blot (B, C) of TCA-precipitated culture supernatants from wild-type *E. coli* O157:H7/NM and antibiotic-resistant mutants. Lanes in panel A: lane 1, molecular weight marker proteins; lane 2, parent 7785; lanes 3–5, three Rif^r 7785 NM derivatives; lane 6, C81 (O157:NM), and lane 7, C57 (O157:H7). In panel B the separated exoprotein fractions were probed with the absorbed immune serum. Lanes: lane 1, molecular weight marker proteins; lane 2, 7785 parent; lane 3, Rif^r non-motile 7785-15 mutant; lane 4, C57; lane 5, C81; and lane 6, EDL933. In panel C the separated exoprotein samples were probed with absorbed immune serum. Lanes: 1. 7785; 2. 7785-15, 3. 7785-A2; 4. C57. FliC is indicated by arrow

The hypermotile Amp^r 7785 mutants produced more FliC protein than parent 7785 or C57 demonstrated by Western blot analysis (Fig. 4C). By using the absorbed immune serum more FliC was observed in the exoprotein fraction of 7785-A2 (lane 3) than in 7785 (lane 1) and C57 (lane 4). The same quantitative difference was observed between parent 7785 and Amp^r mutants in Coomassie brilliant blue strained SDS-PAGE as well (data not shown).

Biochemical profile. To detect any antibiotic resistance mutation associated changes in the fermentation profile of the representative Rif^r and Amp^r mutant strains, 32 main biochemical reactions were determined by API identification program, and sorbitol fermentation was tested on sorbitol-containing MacConkey agar plates. No differences could be observed: the Rif^r mutant strains 7785-5, 7785-15 and Amp^r 7785-A2 gave the same biochemical profile as the parent strain. Parent 7785 was a sorbitol non-fermenting strain and neither the Rif^r nor the Amp^r mutant strains fermented sorbitol after incubation for 24 h.

Phage growth phenotypes of Rif^r and Amp^r mutants. To find additional phenotypic changes parent and Rif^r and Amp^r mutants were examined with *E. coli* and *Shigella sonnei* typing phages in routine test dilution, and the lytic patterns were compared. Several out of the 30 *E. coli* typing phages lysed parent 7785 and EDL933 and none of the 10 *S. sonnei* typing phages lysed these parent O157:H7 strains. Phenotypic changes were observed among the antibiotic-resistant mutant derivatives. In addition to these lytic *E. coli* phages the antibiotic-resistant mutants supported the growth of one or two further phages. Rif^r mutant strains 7785-5 and 933-3 supported the growth of one additional phage, *E. coli* Φ4. This phage did not lyse the parent O157:H7 strains. Rif^r non-motile strain 7785-15 and all the three hypermotile Amp^r mutant strains supported the growth of *S. sonnei* phage ΦVIII in addition to *E. coli* phage Φ4. These changes may be caused by bacterial cell surface modification.

Growth phenotypes of Rif^r and Amp^r mutants. To examine changes in growth phenotypes the permissive temperature and generation times were determined. The growth of parent 7785 and antibiotic-resistant mutants 778-5, 7785-15 and 7785-A2 was examined on LB agar and on MacConkey sorbitol agar plates at different temperatures. The wild-type and all the antibiotic-resistant mutants were able to form colonies at 20, 37 and 42 °C.

The growth rate of the parent 7785, Rif^r mutants 7785-5, 7785-15 and Amp^r mutant 7785-A2 were compared in LB broth at 37 °C. Wild-type 7785 grew faster than 7785-5 and 7785-A2 and slightly slower than 7785-15 (data not shown).

In vivo *virulence.* CFLP mice of 20 g body weight were infected intravenously with 2.5×10^8 colony forming units (CFU) of parent 7785 *E. coli* O157:H7 and the corresponding antibiotic resistant 7785-5, 7785-15 and 7785A2 derivative bacteria. The *in vivo* virulence results are summarised in Table 2. As can be seen, the non-motile 7785-15 and the hypermotile 7785-A2 derivative strains exhibited significantly higher virulence than the wild-type strain. No difference could, however, be observed in the dynamics of the infection as mice infected with any of the 7785 derivatives died mostly within four days after infection. No mice died in the group infected with the same dose of the *E. coli* K 12 strain (data not shown). The parent 7785 *E. coli* O157:H7 and all the corresponding antibiotic-resistant 7785-5, 7785-15 and 7785A2 mutants had *stx1*, *stx2* and *eae* genes demonstrated by PCR.

Table 2

In vivo virulence of parent 7785 *E. coli* O157:H7 strain and its Rif^r and Amp^r mutants in intravenously infected mice

Strain	Died/infected	% death
7785	30/50	60.0
7785-5	38/50[a]	76.0
7785-15	45/50[b]	90.0
7785-A2	44/50[b]	88.0

[a]no significant difference to 7785; [b]significant difference to 7785: $p < 0.01$

Genotypic examinations

To find any polymorphism in the *fliC* gene of Rif^r and Amp^r mutants Southern hybridisation experiments were carried out, and the PCR-amplified entire *fliC* genes of parent 7785 and Rif^r 7785-5, 7785-15 and Amp^r 7785-A2 derivatives were also sequenced and compared.

Southern hybridisation. In Southern blot analysis the genomic DNA of the studied strains was digested with *Bam*HI or *Hin*fI enzyme and probed with K-12 specific *fliC* amplicon. The size of the K-12 specific *fliC* amplicon is 543 bp and it corresponds to the N-terminal and the variable region of the gene. The probe equally hybridised to all of the *E. coli* O157 strains including parent 7785, two corresponding Rif^r mutants 7785-5 and 7785-15 and the O157:NM wild-type strain C81. No polymorphism could be seen between the parent and the mutants or C81 when their genomic DNAs were digested with either *Bam*HI, having one site in the *fliC* gene of *E. coli* K-12 and none in *fliC* gene of EDL933 (Fig. 5) or *Hin*fI, which has seven cutting sites on the *fliC* gene (data not shown). The *fliC*-specific sequences were located on same-sized fragments in all *E. coli* O157 strains tested, regardless of motility. The *fliC*-specific probe hybridised to two different-sized fragments in *E. coli* K-12 strains.

Nucleotide sequence of the E. coli *O157* fliC *gene.* The entire *fliC* gene (1764 bp) of the 7785 and Rif^r 7785-5, 7785-15 and Amp^r 7785-A2 mutants was amplified by PCR using primers described by Fields et al. (1997) and sequenced. As these primers directly meant the first 21 and the last 19 bases of the *fliC* gene for parent 7785 and the corresponding antibiotic-resistant derivatives, we did not determine the first and the last 25 nucleotides, but with the help of further two primers designed for the inner parts of the gene we determined further nucleotide sequence of the *fliC* gene. Polymorphism did not occur, the sequenced 1714 nucleotides were fully identical in all four strains, and comparison with the *fliC* of EDL933 (accession number AE005415) showed 100% identity.

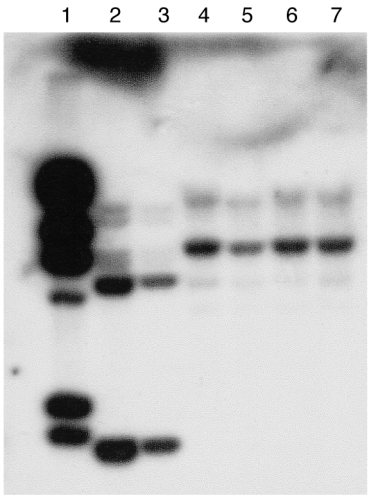

Fig. 5. Southern blot hybridisation of K-12 and O157:H7/H$^-$ strains. Genomic DNA samples were digested with *Bam*HI, electrophoresed in 0.8% agarose gel and probed with K-12 (*E. coli* C600) specific *fliC* amplicon. The hybridising solution contained *Hin*dIII digested λ phage DNA as well. Samples were the following: 1. *Hin*dIII digested λ phage DNA; 2. *E. coli* C600; 3. *E. coli* J5-3; 4. C81; 5. 7785; 6. 7785-15; 7. 7785-5

Discussion

E. coli O157:H7, the prototype of EHEC, is an intensively studied pathogenic agent of great importance. The full genomic sequence of the microorganism is known (Perna et al., 2001). There are two different clones of EHEC, one in the USA – sorbitol non-fermenting motile *E. coli* O157:H7 (Griffin and Tauxe, 1991) – and another type in continental Europe, the sorbitol-fermenting O157 non-motile variety (Karch and Bielaszewska, 2001). Recently the sorbitol non-fermenting and non-motile variant has also been isolated more frequently, but no polymorphism could be observed in the *fliC* gene of strains belonging to *E. coli* O157:H7 and O157:NM serotypes by PCR-RFLP analysis reported by Field et al. (1997). Lately Reid et al. (1999) showed that the *fliC* gene of sorbitol-fermenting O157 strains accumulated multiple mutations, presumably as a result of silencing of flagellin expression. Moreover, two insertions were detected in the conserved N-terminal region of the *fliC* gene of a sorbitol-fermenting O157:NM strain. These insertions produced a shift in the reading frame that introduced a premature stop codon.

According to our studies the motility of spontaneous antibiotic-resistant mutant *E. coli* strains has also changed, but in the *fliC* gene no polymorphism was found either by Southern hybridisation or by determination of the nucleotide sequence. The nucleotide sequence of parent 7785 strain and its antibiotic-

resistant mutants was the same, and it proved to be identical with the *fliC* sequence of EDL933.

LeClerc et al. (1996) reported that *E. coli* O157:H7 was a hypermutable microorganism: 1% of the mutants isolated with rifampicin selection have become resistant to spectinomycin and nalidixic acid. In the present study we experienced no resistance against other antibiotics: there was no resistance against spectinomycin or nalidixic acid. At the same time, however, motility, flagellin production, growth rate, sensitivity towards certain phages and the virulence of Rif^r mutant strains have been changed. Pleiotropic effect is often the result of *rpoB* mutation (Jin and Gross, 1989), which most frequently determines rifampicin resistance (Jin and Gross, 1988). Earlier Yamamori et al. (1977) described nitrosoguanidine-induced Rif^r mutation associated pleiotropic phenotypes in *E. coli* K-12. These Rif^r mutants were isolated at 30 °C and became resistant to phage χ, they were defective in motility and unable to grow at 42 °C. The phage sensitivity of 7785-5 and 7785-15 has been changed as well, but these Rif^r mutant *E. coli* O157 strains were able to grow at 42 °C. However, the antibiotic-resistant mutant 7785-5 and 7785-A2 strains grew slower in LB at 37 °C than did wild-type 7785 (data not shown).

Interestingly, the Amp^r mutant *E. coli* O157:H7 strains also showed pleiotropic phenotypes including a novel hypermotile phenotype. All the three Amp^r mutant strains produced more FliC flagellin protein than the parent 7785 strain demonstrated with SDS-PAGE and immunoblot analysis.

In correlation with several reports we also observed relationship between motility and virulence of the strains. Morooka et al. (1985) demonstrated that mutagenised non-motile *Campylobacter jejuni* derivative strains became defective in colonising the intestinal tract of suckling mice. Feldman et al. (1998) reported that the flagella are essential in the pathogenesis of *Pseudomonas aeruginosa* pulmonary infection. Gardel and Mekalanos (1996) have also observed aberrant virulence factor expression in non-motile and hypermotile *Vibrio cholerae*. Farris et al. (1998) reported that in enteropathogenic *E. coli* (EPEC) BipA, a tyrosine-phosphorylated GTPase protein, there is a virulence regulator, and *BipA* also controls flagella-mediated cell motility to a human host defence protein. Sperandio et al. (1999) reported that genes involved in the formation of attaching and effacing (AE) lesions in EHEC and EPEC were regulated by quorum sensing through autoinducer-2, which is under the control of the *luxS* gene described by Surette et al. (1999). Recently Sperandio et al. (2001) demonstrated that quorum sensing is a global regulatory system since approximately 10% of the genes were regulated by *luxS*. *luxS* up-regulated the expression of flagella, motility and chemotaxis, but down-regulated the transcription of genes encoding Shiga toxin 2 (Stx2), and the *luxS* mutants grew faster than the wild-type strain. In line with these data, in our study the phenotypic changes included modified motility, flagellin production, generation time and virulence. However, in our study the

wild-type 7785 strain was less virulent than the antibiotic resistant derivatives. It can be assumed that expression of flagella- and virulence factor genes is under the control of a regulatory cascade, which simultaneously affects the expression of these different traits. Further investigations are needed to elucidate if *lux*S or other gene(s) are involved in the pleiotropic changes presented in this study.

To the best of our knowledge there is no published paper about rifampicin resistance among wild-type *E. coli* O157 strains. Though rifampicin is not regarded as a therapeutic agent in the treatment of diseases caused by *E. coli* O157, our results give a model for the loss of H7 antigen in association with Rif-resistance mutation in *E. coli* O157:H7 strains.

In summary, in the present study we described rifampicin-resistance mutation associated non-motile and ampicillin-resistance mutation associated hypermotile phenotypes, which developed in *E. coli* O157:H7 by a hitherto unidentified mechanism. Our results suggest that this *E. coli* O157:NM mutant is increased in virulence and has several further new features such as modified growth capacity and altered phage sensitivity. Further investigation is needed to elucidate if *luxS* directed functions or other regulatory mechanisms are involved in the phenotypic changes observed in our mutants.

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