Genomic analysis of a multidrug-resistant strain of enterohaemorrhagic *Escherichia coli* O157: H7 causing a family outbreak in Japan

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A family outbreak of enterohaemorrhagic *Escherichia coli* (EHEC) O157: H7 infection occurred in October 2003 in the Hiroshima prefecture, Japan. Four isolates of EHEC O157: H7, 03064, 03065, 03066 and 03067, were recovered from a 1-year-old daughter, mother, father and 3-year-old daughter, respectively. All EHEC O157: H7 isolates were positive for Stx1 and Stx2 Shiga toxins. Surprisingly, DNA fingerprinting profiles obtained by PFGE showed that the first isolate, 03064, had unique XbaI and BlnI profiles that differed from the other three isolates. Also, plasmid analysis results revealed that isolate 03064 contained an extra plasmid larger than the classic large plasmid of EHEC O157, pO157 (93.6 kb). This new plasmid was named pMDR157. Furthermore, isolate 03064 showed a multidrug-resistance (MDR) phenotype against streptomycin, spectinomycin, co-trimoxazole (trimethoprim/sulfamethoxazole), ampicillin and tetracycline; the other isolates were completely sensitive to these antibiotics. Molecular analysis of the MDR phenotype in this unique strain revealed the presence of a class 1 integron containing two gene cassettes: a dihydrofolate reductase type 1 gene (*dfrI*), which confers resistance to trimethoprim, and an aminoglycoside adenyltransferase gene (*aadA1*), which confers resistance to streptomycin and spectinomycin. Southern blot hybridization showed that the class 1 integron was located in the extra plasmid, pMDR157. The ampicillin resistance was found to be due to the presence of the TEM-1-type β-lactamase gene. The MDR phenotype was transferred successfully to *E. coli* HB101 by conjugation, indicating that both the class 1 integron and the TEM-1 β-lactamase were located on the conjugative transferable plasmid, pMDR157. To the authors’ knowledge, this is the first report of the identification of a β-lactamase gene in EHEC O157.

INTRODUCTION

Enterohaemorrhagic *Escherichia coli* (EHEC) O157: H7 is considered one of the most important recently emerged food-borne pathogens. It causes diarrhoea that may result in life-threatening complications such as haemorrhagic colitis and haemolytic uraemic syndrome (Paton & Paton, 1998). It has been responsible for sporadic and epidemic gastrointestinal infections worldwide (Jay *et al.*, 2004; Nataro & Kaper, 1998). Infection with EHEC O157 causes an estimated 73 000 illnesses and 60 deaths annually in the USA (Mead *et al.*, 1999). In Japan, a severe outbreak of haemorrhagic colitis and haemolytic uraemic syndrome associated with EHEC O157: H7 occurred in 1990 (Akashi *et al.*, 1994). Later, more than 10 000 cases of EHEC O157: H7 infection were reported from May to August 1996 (National Institute of Infectious Diseases, 1997). Initial clusters of that outbreak occurred in the Hiroshima and Okayama prefectures and an extraordinarily large outbreak affected the Osaka prefecture in the city of Sakai in July 1996. This epidemic is considered the largest outbreak of EHEC O157 reported thus far. In 1999, another outbreak was reported in Kyoto city (Watanabe *et al.*, 1999). According to the recent report of National Epidemiological...
Surveillance of Infectious Diseases (NESID) Japan, 1616 symptomatic and 1370 asymptomatic (a total of 2986) new cases of EHEC infection were notified in 2003 (National Institute of Infectious Diseases, 2004).

Recently, MDR phenotypes have spread widely among Gram-negative bacteria. A genetic element known as an integron may be one of the most important factors involved in this spread of genetic material (Jones et al., 1997). Integrons are potentially mobile genetic elements frequently located on transposons or conjugative plasmids that can serve as vehicles for the intra- and interspecies transmission of genetic material. Class 1 integrons are most frequently found among clinical isolates, and their structure consists of two conserved segments (5’- and 3’-CS) and an internal variable region that contains gene cassettes encoding antimicrobial-resistance determinants (Rowe-Magnus & Mazel, 2001). In Japan, we recently characterized class 1 integrons in enterotoxigenic Escherichia coli (ETEC) O159 (Ahmed & Shimamoto, 2004) and enteroinvasive Escherichia coli (EIEC) O164 (Ahmed et al., 2005).

The severity of disease, the lack of effective treatment and the potential for large-scale outbreaks from contaminated food supplies have propelled intensive research into the pathogenesis and detection of EHEC O157. Chemotherapy for Shiga toxin-producing E. coli (STEC) infections remains controversial; although some studies do not advise antibiotic treatment for infections caused by E. coli O157 in humans (Wong et al., 2000), others suggest that disease progression may be prevented by administering antibiotics at an early stage (Takeda et al., 1997; Shiomi et al., 1999). Recent reports have determined the antibiotic resistant phenotypes of EHEC O157 (Bettelheim et al., 2003; Khan et al., 2002; Vali et al., 2004); however, there are few reports related to the molecular basis of these resistances (Morabito et al., 2002; Zhao et al., 2001). Hence, the goal of this study was to analyse the genomic DNAs of the four isolates of EHEC O157: H7 isolated from the previously mentioned family, and to characterize the molecular basis of the MDR phenotype found in the first isolate, 03064.

**METHODS**

**Bacterial strains and patients.** EHEC O157: H7 isolates 03064, 03065, 03066 and 03067 were isolated from four Japanese family members, a 1-year-old daughter, mother, father and 3-year-old daughter, respectively, in October 2003 in the Hiroshima prefecture, Japan. All family members except the father were suffering from diarrhoea. EHEC O157:H7 strains were identified by standard procedures (Bopp et al., 1999). Serological typing for O157 and H7 antigens was performed by slide agglutination with polyvalent and monovalent anti-E. coli O and H sera according to manufacturer’s instructions. Rifampicin-resistant E. coli HB101 (Rif) was used as a recipient strain for conjugation experiments (Ahmed et al., 2005).

**Antimicrobial susceptibility testing.** EHEC O157: H7 isolates 03064, 03065, 03066 and 03067 were tested for susceptibility to many antibiotics including ampicillin (10 μg), chloramphenicol (30 μg), ciprofloxacin (5 μg), kanamycin (30 μg), gentamicin (10 μg), nalidixic acid (30 μg), streptomycin (10 μg), co-trimoxazole (trimethoprim and sulfamethoxazole) (20 μg), tetracycline (30 μg) and cefotaxime (5 μg) using the disc diffusion method. Subsequently, the MICs of these antibiotics and spectinomycin were determined for the multidrug-resistant EHEC O157: H7 isolate 03064 using the NCCLS broth microdilution method. MIC breakpoints were evaluated according to NCCLS guidelines (NCCLS, 2002).

**PFGE analysis.** All isolates of EHEC O157: H7 were analysed by PFGE with the restriction enzymes XbaI and BglI using standardized methods (Centers for Disease Control and Prevention, 2000). The DNA was separated by PFGE using the GenePath system (Bio-Rad). Following electrophoresis, the gels were stained with 0.5 μg ethidium bromide ml−1, illuminated under UV light and photographed.

**Plasmid isolation.** Plasmid DNA was extracted from all EHEC O157: H7 isolates according to the method of Kado & Liu (1981) for large plasmids.

**Bacterial DNA preparation, PCR and DNA sequencing.** The preparation of the bacterial DNA template and PCR conditions for the detection of the class 1 integron were carried out as described elsewhere (Zhao et al., 2001). The class 1 integron primers 5’-CS and 3’-CS (Table 1) (Lévesque et al., 1995), which amplify the region between 5’-CS and 3’-CS, were used. Additionally, the multidrug-resistant EHEC O157: H7 isolate 03064 was tested for the β-lactamase-encoding genes TEM, SHV and OXA by PCR as described elsewhere (Weill et al., 2004). Universal primers for the TEM, SHV and OXA families were used (Table 1). The reaction products of PCR were subjected to electrophoresis in a 1% agarose gel, stained with ethidium bromide and visualized under UV light. The PCR fragment was then purified from the agarose gel using a QIA quick gel extraction kit (QIAGEN). The PCR products were sequenced using an ABI automatic DNA sequencer (model 373, Perkin-Elmer).

**Southern blot hybridization.** Plasmids were isolated from EHEC O157: H7 isolates 03064 and 03065 by using QIAGEN large-construct kits according to the manufacturer’s instructions. After agarose gel electrophoresis, the DNA fragments in the gel were transferred onto Hybond-N+ nylon membranes (Amersham Biosciences) according to the manufacturer’s instructions. The DNA fragment containing the whole class 1 integron was amplified by PCR using the integron primers 5’-CS and 3’-CS (Table 1) and purified as described above. The purified fragment was labelled with alkaline phosphatase using the AlkPhos Direct Labelling System (Amersham Biosciences), and subsequently used as a DNA probe. All hybridization steps were carried out according to the manufacturer’s protocol. The hybridization was performed at 55 °C for 12 h, and the hybridized DNA was detected using the CDP-Star chemiluminescent signal generation system (Amersham Biosciences) according to the manufacturer’s instructions.

**Conjugation experiments.** Direct transfers of the extra plasmid carrying resistance genes were performed by mating the donor strain, EHEC O157: H7 isolate 03064, with a rifampicin-resistant mutant of E. coli HB101 obtained in vitro (Sambrook & Russell, 2001) as the recipient strain at 37 °C in solid and liquid Mueller–Hinton media. Transconjugants were selected on Mueller–Hinton agar containing 200 μg rifampicin ml−1, 100 μg ampicillin ml−1, 50 μg streptomycin ml−1 and 25 μg trimethoprim ml−1.

**Computer analysis of the sequence data.** A similarity search was carried out using the BLAST program available at the NCBI BLAST homepage (http://www.ncbi.nlm.nih.gov/blast/).
RESULTS AND DISCUSSION

EHEC O157 as a global public health concern

The recently recognized pathogen E. coli O157: H7 appears to be increasingly causing severe and potentially life-threatening illness (Paton & Paton, 1998). It causes a range of illnesses including non-bloody diarrhea, haemorrhagic colitis and haemolytic uremic syndrome. Haemolytic uraemic syndrome is the main cause of acute renal failure in children (Cordovez et al., 1992). In 1983 the first outbreak of gastrointestinal illness due to EHEC O157 infection was reported (Riley et al., 1983). Later on, EHEC O157 was found to be responsible for numerous food- and water-borne outbreaks especially in Japan, the USA and Europe. (Akashi et al., 2004).

In Japan, infection with EHEC is listed as a category III notifiable infectious disease under the law concerning the Prevention of Infectious Diseases and Medical Care for Patients of Infectious Diseases (National Institute of Infectious Diseases, 2004). In this study we report a family outbreak due to EHEC O157: H7 infection. Four isolates of EHEC O157: H7, 03064, 03065, 03066 and 03067, were recovered from family members. The outbreak began with the 1-year-old daughter, followed by her mother, father and finally her 3-year-old sister. Recently, a similar family outbreak due to EHEC O157 was reported in Slovakia (Liptakova et al., 2004).

Antimicrobial-resistance phenotype

The disc diffusion test was used to check the antimicrobial phenotypes of the four isolates of EHEC O157: H7. Surprisingly, only the 03064 isolate showed an MDR phenotype, and the other isolates were completely sensitive for all tested antibiotics. EHEC O157: H7 isolate 03064 showed resistance to streptomycin, spectinomycin, co-trimoxazole (trimethoprim/sulfamethoxazole), ampicillin and tetracycline. This MDR phenotype was further confirmed by determination of MICs for these antibiotics as follows: streptomycin, 32 µg ml⁻¹; spectinomycin, 64 µg ml⁻¹; ampicillin, 128 µg ml⁻¹; co-trimoxazole (trimethoprim/sulfamethoxazole), 64/608 µg ml⁻¹; and tetracycline, 64 µg ml⁻¹. Similar resistance phenotypes for other EHEC O157 isolates have been reported recently (Bettelheim et al., 2003; Khan et al., 2002; Vali et al., 2004).

Genomic analyses

The sharp difference in the antimicrobial-resistance phenotypes between the first isolate (03064) and the other three isolates (03065, 03066 and 03067) indicated that the MDR phenotype associated with the first isolate must be encoded by a mobile genetic element that may have been lost during transmission of EHEC O157: H7 to the other family members. Hence, genomic DNA from the four EHEC O157: H7 isolates was examined by PFGE and plasmid analysis.

The genomic DNA of the four EHEC O157: H7 isolates was analysed by PFGE after treatment with the restriction enzymes XbaI and BlnI. Strikingly, PFGE results showed that the first isolate, 03064, had unique XbaI and BlnI profiles that differed from the other three isolates. It had one extra band with the XbaI enzyme (Fig. 1a) and an extra two bands with the BlnI enzyme (Fig. 1b). Interestingly, similar PFGE findings for other related clinical isolates with different PFGE patterns have previously been reported. For example, Harnett et al. (1997) reported PFGE pattern variation between the post-treatment isolate and the original isolate of Neisseria gonorrhoeae. Also, one band difference in PFGE patterns between two isolates from the same patient has been reported elsewhere (Su & Lind, 2001).

It is well known that most of the clinical isolates of EHEC O157: H7 harbour a large virulence plasmid designated pO157 (Nataro & Kaper, 1998). The molecular size of pO157 is 93·6 kb (Schmidt et al., 1996). A 3·4 kb fragment of this plasmid was subsequently shown to encode enterohaemolysin. A number of other smaller plasmids ranging in size from 2 to 87 kb have been found in strains of E. coli O157: H7 (Nataro & Kaper, 1998). In this study, plasmid analysis results showed that all the isolates had pO157 (Fig. 1c). Surprisingly, the first isolate (03064) had one extra plasmid larger than pO157 (Fig. 1c). This extra plasmid was named pMDR157. It may be responsible for the MDR phenotype and the extra bands of the PFGE profiles of EHEC O157 isolate 03064.

Table 1. Oligonucleotides

<table>
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<tr>
<th>Primer name</th>
<th>Nucleotide sequence (5’–3’)</th>
<th>Target size</th>
<th>Reference/GenBank accession no.</th>
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<tr>
<td>5′-CS</td>
<td>GGCATCAAAGCAGCAAG</td>
<td>Variable</td>
<td>Lévesque et al. (1995)</td>
</tr>
<tr>
<td>3′-CS</td>
<td>AAGCAAGCTTGGACCTGA</td>
<td>1080 bp</td>
<td>Weill et al. (2004)</td>
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<tr>
<td>TEM-F</td>
<td>ATAAATTTCTTGAAGACGAAA</td>
<td>795 bp</td>
<td>Weill et al. (2004)</td>
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<tr>
<td>TEM-R</td>
<td>GACGTATCAATGCTTAAATC</td>
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<tr>
<td>SHV-F</td>
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<td>SHV-R</td>
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<tr>
<td>OXA-R</td>
<td>GTGTGTGTGAAATGTTGTA</td>
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Antimicrobial-resistance phenotype

The disc diffusion test was used to check the antimicrobial phenotypes of the four isolates of EHEC O157: H7. Surprisingly, only the 03064 isolate showed an MDR phenotype, and the other isolates were completely sensitive for all tested antibiotics. EHEC O157: H7 isolate 03064 showed resistance to streptomycin, spectinomycin, co-trimoxazole (trimethoprim/sulfamethoxazole), ampicillin and tetracycline. This MDR phenotype was further confirmed by determination of MICs for these antibiotics as follows: streptomycin, 32 µg ml⁻¹; spectinomycin, 64 µg ml⁻¹; ampicillin, 128 µg ml⁻¹; co-trimoxazole (trimethoprim/sulfamethoxazole), 64/608 µg ml⁻¹; and tetracycline, 64 µg ml⁻¹. Similar resistance phenotypes for other EHEC O157 isolates have been reported recently (Bettelheim et al., 2003; Khan et al., 2002; Vali et al., 2004).
Molecular analysis of the MDR phenotype of EHEC O157 isolate 03064 using PCR and DNA sequencing revealed the presence of a class 1 integron of 1552 bp. This class 1 integron (Fig. 2) contained two gene cassettes: (1) a dihydrofolate reductase type 1 gene, *dfrI*, which confers resistance to trimethoprim, and (2) an aminoglycoside adenyltransferase gene type 1, *aadA1*, which confers resistance to streptomycin and spectinomycin. To date, there are only a few reports that document the presence of class 1 integrons in EHEC O157 (Morabito et al., 2002; Zhao et al., 2001). Additionally, the use of a set of primers for the conserved regions of common \( \beta \)-lactamase genes (Table 1), showed that the resistance of the EHEC O157 isolate 03064 to ampicillin was due to the presence of the TEM-1-type \( \beta \)-lactamase gene. To our knowledge, this TEM-1-type \( \beta \)-lactamase gene is the first recorded \( \beta \)-lactamase gene in EHEC O157.

**Southern blot hybridization and transfer of the MDR phenotype**

Southern hybridization was used to determine the location of the class 1 integron in *E. coli* O157 : H7 isolate 03064 in comparison to isolate 03065. Many plasmids of different sizes were detected in both isolates (Fig. 3), but a positive hybridization signal was detected only in the extra plasmid, pMDR157, of *E. coli* O157 : H7 isolate 03064 (Fig. 3).

In order to confirm that the MDR phenotype of the first *E. coli* O157 : H7 isolate 03064 was encoded on a conjugative transferable plasmid, pMDR157, a conjugation experiment was carried out between EHEC O157 : H7 isolate 03064 as a donor and a rifampicin-resistant mutant of *E. coli* HB101 as a recipient. After conjugation, the MDR phenotype was successfully transferred to *E. coli* HB101, indicating that the MDR determinant genes are present on pMDR157 of *E. coli* O157 : H7 isolate 03064, and that this plasmid is transferable. These results could explain the role of the extra plasmid in the MDR phenotype of *E. coli* O157 : H7 isolate 03064. This extra plasmid was isolated from the first patient and lost during the transmission of the same clone of O157 : H7 to the other family members.

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**Fig. 1.** Analysis of the genomic DNA from EHEC O157 : H7 isolate 03064 (lane 1), isolate 03065 (lane 2), isolate 03066 (lane 3), and isolate 03067 (lane 4). Lanes M are size markers (λ phage ladders). (a, b) PFGE profiles of the genomic DNA digested with restriction enzymes Xba I (a) and Bln I (b). The large arrows indicate the extra PFGE bands present only in the EHEC O157 : H7 isolate 03064. (c) Plasmid profiles for the EHEC O157 : H7 isolates. The small arrow indicates the classic plasmid of EHEC O157 : H7, pO157 (93.6 kb), present in all isolates; the large arrow indicates the additional large plasmid (pMDR157) of EHEC O157 : H7 isolate 03064 (lane 1) carrying the antimicrobial-resistance genes.

**Fig. 2.** Organization of the class 1 integron of EHEC O157 : H7 isolate 03064. Black boxes, 5′- and 3′-conserved sequences. Hatched arrows, antibiotic gene cassettes of the dihydrofolate reductase type 1 gene (*dfrI*), which confers resistance to trimethoprim, and aminoglycoside adenyltransferase type 1 gene (*aadA1*), which confers resistance to streptomycin and spectinomycin. Hatched circles, the 59-base elements (59-be) of the gene cassettes.
Conclusion

In this study we analysed the genomic DNA of a multidrug-resistant clone of EHEC O157: H7 that caused a family outbreak in Japan. We also characterized the molecular basis of the MDR phenotype in this strain and identified a β-lactamase gene in EHEC O157. This study provides further insight into the role of mobile genetic elements in MDR phenotypes in food-borne pathogenic bacteria.

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REFERENCES


