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Carriage of Mobilizable Plasmid-Mediated β-Lactamase Gene in Ampicillin-Resistant *Escherichia coli* Strains with Origin of Normal Fecal Flora

Osman Birol ÖZGÜMÜŞ[1]
İlknur TOSUN[2]
Faruk AYDIN[2]
Ali Osman KILIÇ[2]
Murat ERTÜRK[2]

[1] Department of Biology, Rize Faculty of Arts & Sciences, Karadeniz Technical University, 53050 Rize - TURKEY

[2] Department of Microbiology and Clinical Microbiology, Faculty of Medicine, Karadeniz Technical University, 61080 Trabzon - TURKEY

Aim: The aim of this study was to investigate the carriage of β-lactamase genes in ampicillin-resistant (Amp^r) *Escherichia coli* (*E. coli*) isolates from human normal fecal flora.

Methods: Ten Amp^r *E. coli* strains isolated from the stool samples of 21 healthy persons with no antibiotic use during at least three months were screened for TEM-, SHV-, or OXA-type β-lactamase genes by polymerase chain reaction (PCR). The susceptibility of the strains to antibiotics was determined by disk diffusion method, and minimum inhibitory concentration (MIC) of ampicillin to the strains was determined by agar dilution method. Plasmid transfer assays were performed by broth mating technique. Plasmid DNA was isolated by alkaline lysis method. Digoxigenin-labeled TEM-1 probe was used in hybridization assays.

Results: Two of 10 strains were found to be carrier for only TEM-type β-lactamase gene (bla_{TEM}) by PCR, and their resistances to ampicillin were conjugatively transferred to a recombinant *E. coli* K-12 strain C600. MIC of ampicillin to two representative strains and their transconjugants was detected as >512 μg/ml. Moreover, β-lactamase inhibitor resistance was also observed in these two strains and their transconjugants. Digoxigenin-labeled TEM-1 DNA probe was hybridized to some non-conjugative but mobilizable plasmid DNAs purified from two of the TEM-gene-carrying organisms.

Conclusions: These results indicate that commensal *E. coli* strains carrying β-lactamase gene in the bowel environment could retain resistance determinants on small-sized resistance plasmids (R plasmids) and become a potential reservoir for resistance genes in the community, even in the absence of recent antibiotic consumption.

Key Words: Fecal flora, ampicillin-resistant *Escherichia coli*, mobilizable β-lactamase gene

Normal Fekal Flora Kökenli Ampisiline Dirençli *Escherichia coli* Suşlarında Taşınabilir Plazmit Aracılı β-laktamaz Gen Taşıyıcılığı

Amaç: Bu çalışmanın amacı, insanın normal fekal florasından izole edilen ampisiline dirençli (Amp^r) *Escherichia coli* izolatlarında β-laktamaz genlerinin taşıyıcılığının araştırılmasıydı.

Yöntemler: En az üç ay boyunca antibiyotik kullanmamış 21 sağlıklı kişinin dışkı örneklerinden izole edilen 10 Amp^r *E. coli* suşu TEM-, SHV-, ve OXA-tipi β-laktamaz genleri açısından polimeraz zincir reaksiyonu (PZR) ile tarandı. Suşların antibiyotiklere hassasiyetleri disk difüzyon metodu ile, ampisilinin suşlara karşı minimum inhibitör konsantrasyonu (MİK) agar sulandırım metodu ile belirlendi. Plazmit aktarma deneyleri sıvıda çiftleşme metodu ile yapıldı. Plazmit DNA'sı alkali lizis tekniği ile izole edildi. Hibridizasyon deneylerinde digoxygenin ile işaretli TEM-1 probu kullanıldı.

Bulgular: On suşun ikisinin PZR ile yalnızca TEM-tipi geni (bla_{TEM}) taşıyıcısı olduğu bulundu ve ampisiline karşı dirençleri konjugatif olarak bir rekombinant *E. coli* K-12 suş C600'e aktarıldı. Ampisilinin iki orijinal suşa ve transkonjugantlarına karşı MİK'leri >512 μg/mL olarak tespit edildi. Ayrıca, bu iki suş ve transkonjugantlarında β-laktamaz inhibitör direnci de gözlendi. Digoxygenin ile işaretli TEM-1 DNA probu TEM-geni taşıyan iki organizmadan izole edilmiş olan bazı non-konjugatif fakat taşınabilir plazmit DNA'larına hibridize oldu.

Sonuç: Bu sonuçlar; barsak ortamında β-laktamaz geni taşıyan kommensal *E. coli* suşlarının direnç determinantlarını küçük direnç plazmitleri (R plazmit) üzerinde taşıyabildiğini ve yakın zamanda antibiyotik kullanılmasa dahi bunların toplumda direnç genlerinin potansiyel bir rezervuarı haline gelebileceğini göstermektedir.

Anahtar Sözcükler: Fekal flora, ampisiline dirençli *Escherichia coli*, taşınabilir β-laktamaz geni

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Correspondence
Osman Birol ÖZGÜMÜŞ
Department of Biology,
Faculty of Arts & Sciences,
Rize University,
53050 Rize – TURKEY

ozgumus@ktu.edu.tr

Introduction

Resistance to antibiotics is highly prevalent in bacterial isolates worldwide, especially in developing countries (1,2). The fecal flora represents a potential reservoir for the environments where antibiotic resistance genes can be transferred from the commensal flora to virulent microorganisms (3,4). The prevalence of resistance genes in commensal *Escherichia coli* (*E. coli*) is a useful indicator of resistance genes in bacteria in the community (5-8).

It was reported (9) that high frequencies of antimicrobial resistance have been found in enterobacteria, in fecal flora as well as in clinical isolates, and we know little about how their resistance is acquired and maintained. There are some reports (10,11) that there is a trend of infection by surveillance of the feces, and *E. coli* is the main carrier of antimicrobial resistance genes in fecal flora; resistance in other enterobacterial species is rare in the absence of antimicrobial selection.

Principals of the gene types responsible for conferring resistance to β-lactam antibiotics are plasmid-mediated TEM-, SHV- and OXA-type β-lactamase genes. The enzymes, and most of their gene products, can hydrolyze ampicillin and oxyimino-β-lactams. The commonest of these enzymes in enterobacteria is TEM-1, which is responsible for the ampicillin resistance seen in about 50% of *E. coli* isolates (12).

In this study, carriage of TEM-, SHV-, or OXA-type β-lactamase genes and the genetic elements bearing these genes were investigated in ampicillin-resistant (Amp^r) *E. coli* strains from the stool samples of healthy persons.

Materials and Methods

Fecal samples in the study were collected from the selected 21 healthy persons who had received no antibiotic treatment for at least three months prior to sampling and who presented to the microbiology research laboratory for the purpose of parasitological check-up, at the teaching hospital of Karadeniz Technical University in Trabzon, a rural city settled on the Black Sea coastline in northern Turkey. The subjects were provided with sterile containers for stool collection and were told to bring a fresh sample, preferably collected in the morning on the same day. All samples were cultured on the same day. Four 10-fold dilutions of the stool samples were made in physiological saline, plated onto plates containing eosin-methylene blue (EMB) agar (Oxoid), a medium which is selective for aerobic Gram-negative enteric bacilli, supplemented with 50 µg/ml of ampicillin (Fischer), and incubated aerobically overnight at 35ºC. (According to the criteria of the National Committee for Clinical Laboratory Standards [NCCLS], members of the *Enterobacteriaceae* family capable of growing in a concentration of >32 µg/ml of ampicillin are regarded as resistant to ampicillin (13).

At least five presumptive (according to the colony morphology) *E. coli* colonies on EMB agar (Oxoid) were picked up by a sterile toothpick and replica plated on a fresh EMB medium (Oxoid) containing 50 µg/ml of ampicillin (Fischer). Complete identification of presumptive *E. coli* strains capable of growing in the selection of ampicillin was achieved by use of the tests in Bergey's Manual of Determinative Bacteriology (14) and the conventional methods described by Balows et al. (15). After identification of species level, organisms were stored in Luria-Bertani (LB) broth (1% tryptone, 0.5% yeast extract, 0.5% NaCl, pH 7.4) containing 20% of glycerol (Merck) at -30 ºC until next use.

Conjugation assays were performed by broth mating method (16). Equal volumes (1 ml) of cultures of the Amp^r *E. coli* strains as donor and *E. coli* K12 strain C600 (F^- *thr leu thi* Rif^r) as the recipient, grown with agitation in LB broth were mixed and incubated for 18 h at 35ºC without shaking. Transconjugants were selected on EMB agar (Oxoid) supplemented with 150 µg/ml rifampin (Hoecst) and 100 µg/ml ampicillin (Fischer). The frequency of transfer was expressed relative to the number of donor cells.

The susceptibilities of Amp^r *E. coli* strains and *E. coli* C600 transconjugants to the antibiotics (Oxoid) ampicillin (10 µg), sulbactam/ampicillin (10 µg/10 µg), cefazolin (30 µg) and ceftazidime (30 µg) were determined by the standard disk diffusion method as described in NCCLS guidelines (13). The results were interpreted by using the breakpoints in the same guideline. Antimicrobial susceptibility to ampicillin was also determined by agar dilution method according to the NCCLS (17).

Plasmid DNAs were isolated from the strains by alkaline extraction method (18). Purified DNAs were electrophoresed in 0.9% agarose gel containing 0.5 µg/ml of ethidium bromide (Sigma), and were photographed under UV light.

To prepare templates for polymerase chain reaction (PCR), the strains and their transconjugants were inoculated into 3 mL of LB broth and incubated for 20 h at 37ºC with shaking. Cells from 1.5 mL of the overnight culture were harvested by micro-tube centrifugation. After decanting the supernatant, the pellet was re-suspended in 500 µL of sterile deionized water, and boiled for 10 min. After centrifugation, 1-µL of supernatant was used as template for PCR assays by using the oligonucleotide primers, mixture composition and cycling conditions previously determined by Arlet and Philippon (19) and by Ouellette et al. (20) for bla_{TEM}, bla_{SHV} and bla_{OXA}, respectively. The PCR products were electrophoresed in 2% agarose gel, stained with 0.5 µg/mL of ethidium bromide (Sigma), and photographed with UV illumination.

Plasmid DNAs were transferred onto a nylon membrane by using the method of Southern (21). After the intragenic PCR products of TEM-1 gene, amplified from pUC18 bearing TEM-1 type β-lactamase gene, were labeled with digoxigenin as described by the manufacturer (Bohringer-Mannheim), the blot was hybridized with digoxigenin-labeled intragenic TEM-1 DNA probe to detect the localizations of bla_{TEM} genes. Hybridization was carried out under conditions of high stringency (68ºC) with detection of label by enzyme-linked immunoassay.

Results

The plasmid content analyses (gel photography not shown) of 84 presumptive *E. coli* strains picked up from each of the EMB agar plates on which had been previously spread four 10-fold dilutions of the fecal samples obtained from 21 healthy volunteers revealed only 10 Amp^r *E. coli* strains harboring the plasmids with different DNA band patterns ranging from 0.5 kb to >10 kb in molecular size (Figure 1). Plasmid DNA bands with the same patterns were also purified from the unrelated Amp^r *E. coli* strains (*E. coli* EC1b and *E. coli* EC1c) of the same person, as seen in lane 1 and lane 2 in Figure 1. Except for two (EC1a and EC1c) out of 10 Amp^r *E. coli* strains, the remaining eight (EC2b, EC3, EC4a, EC8, EC9b, EC14b, EC17, EC19) were found to belong to the flora of unrelated persons. Consequently, 10 Amp^r *E. coli* strains were isolated from the nine stool samples obtained from 21 healthy persons, suggesting that Amp^r *E. coli* colonization was determined as nearly 43% in the volunteers (Table 1).

It was observed that ampicillin resistance was transferable in only two *E. coli* strains (EC1c and EC4a) at a frequency of $3 - 4 \times 10^{-7}$ (Table 2). Transconjugants were detected to harbor <10 kb of plasmid DNA bands (gel photography not shown). Phenotypic antibiotic resistance patterns and properties of harbored plasmids

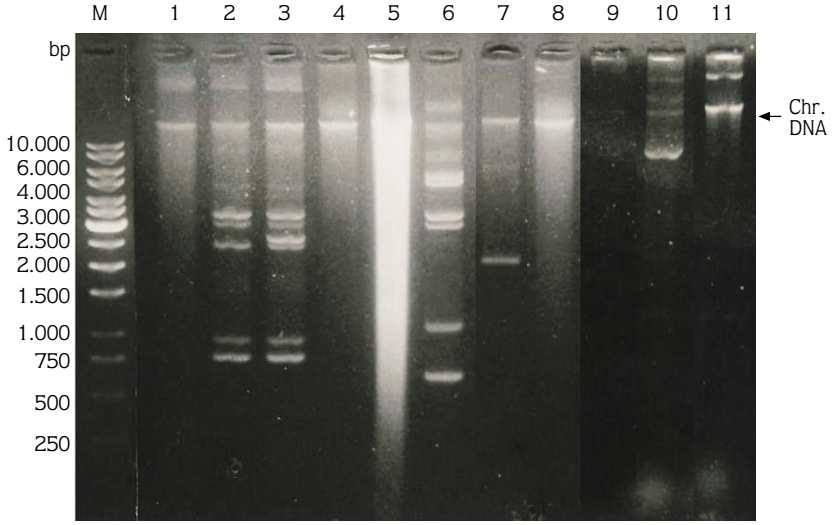

Figure 1. Agarose gel electrophoresis of plasmid DNAs from the fecal ampicillin-resistant *E. coli* strains. M, 1 Kb DNA Ladder (MBI Fermentas, USA); 1, *E. coli* EC1a; 2, *E. coli* EC1b; 3, *E. coli* EC1c; 4, *E. coli* EC2b; 5, *E. coli* EC3; 6, *E. coli* EC4a; 7, *E. coli* EC8; 8, *E. coli* EC9b; 9, *E. coli* EC14b; 10, *E. coli* EC17; 11, *E. coli* EC19.

Table 1. Carriage of plasmid and β-lactamase gene in ampicillin-resistant *E. coli* strains with fecal origin.

Person	Amp^r *E. coli* strain[a]	TEM PCR	SHV PCR	OXA PCR	Plasmid band (~kb)[b]	DNA band hybridized with TEM-1 probe (~kb)
1.	EC1a	-	-	-	>10	-
	EC1c	+	-	-	>10, 7.3, 4.5, 3.4, 2.5, 0.9, 0.7	7.3, 3.4, 2.5
2.	EC2b	-	-	-	(-)	-
3.	EC3	-	-	-	(-)	-
4.	EC4a	+	-	-	>10, 7.3, 4.5, 3, 1.2, 0.5	4.5, 3
5.	EC8	-	-	-	2	-
6.	EC9b	-	-	-	(-)	-
7.	EC14b	-	-	-	(-)	-
8.	EC17	-	-	-	>10, >10, 7.5	-
9.	EC19	-	-	-	>10	-

[a] E. coli EC1a and EC1c are the unrelated strains isolated from the same person.
[b] (-), Plasmid DNA band not determined.

Table 2. Properties of bla_{TEM}-bearing donor strains and R$^+$ C600 transconjugants.

Amp^r *E. coli* strain	Antibiotic resistance phenotype[a,b]		Plasmid content of R$^+$ C600 (~kb)[c]	Transfer frequency
	Donor	R$^+$ C600		
EC1c	Amp Sam Cz	Amp Sam Cz	?, 2.5	$3x10^{-7}$
EC4a	Amp Sam Cz	Amp Sam Cz	?, 3	$4x10^{-7}$

[a] Amp: ampicillin. Sam: ampicillin/sulbactam. Cz: cefazolin.
[b] MIC value of ampicillin to donor strains and their transconjugants was detected as >512 µg/ml.
[c] ?: Self-transmissible cryptic plasmid.

of these two strains are shown in Table 2. In susceptibility testing performed by agar dilution method, ampicillin showed inhibitory effect on two strains (EC1c and EC4a) and their R$^+$ transconjugants in MIC of >512 µg/mL. These strains were also detected to be inhibitor-resistant, and they were resistant to ampicillin/sulbactam and cefazolin, but susceptible to ceftazidime according to the disk diffusion test.

In PCR assays performed for the carriages of bla_{TEM}, bla_{SHV} and bla_{OXA} of the Amp^r *E. coli* strains, only bla_{TEM} genes were detected in two strains (EC1c and EC4a), as seen in Table 1. 504-bp intragenic PCR products of TEM-genes can be seen on agarose gel electrophoresis in Figure 2.

In TEM-specific hybridization assay of the plasmid DNAs (Figure 3A) from TEM-gene-bearing organisms, digoxigenin-labeled intragenic TEM-1 DNA probe was hybridized to the 7.3 kb-, 3.4 kb- and 2.5 kb-DNA bands in *E. coli* EC1c, and to the 4.5 kb- and 3 kb-DNA bands in *E. coli* EC4a (Figure 3B).

Discussion

Ampicillin, to which a considerable rise in resistance was seen from the middle of the 1980s to the end of the 1990s, is extensively used in developing countries (2,22). Our results showed that nine (nearly 43%) of 21 healthy volunteers were detected to carry Amp^r *E. coli,* in which the resistances were confirmed chromosomally and/or by

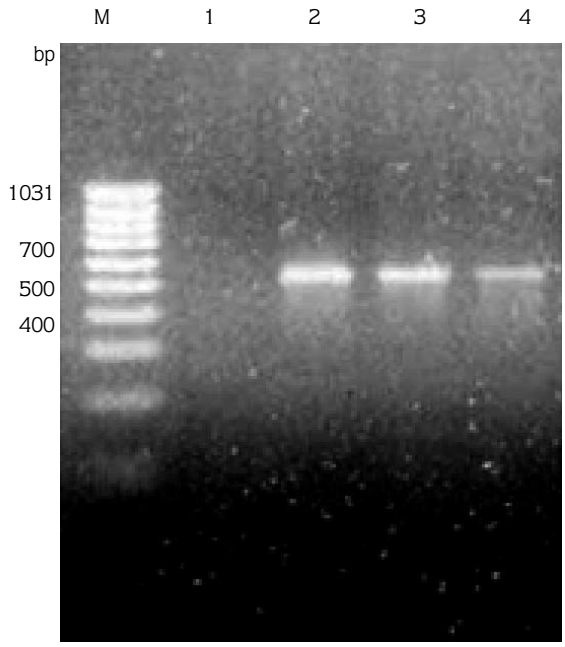

Figure 2. PCR analysis of bla_{TEM} genes in ampicillin-resistant *E. coli* strains. M, 100 bp DNA Ladder (MBI Fermentas, USA); 1, *E. coli* K12 C600 (negative control); 2, pUC18 (positive control); 3, *E. coli* EC1c; 4, *E. coli* EC4a.

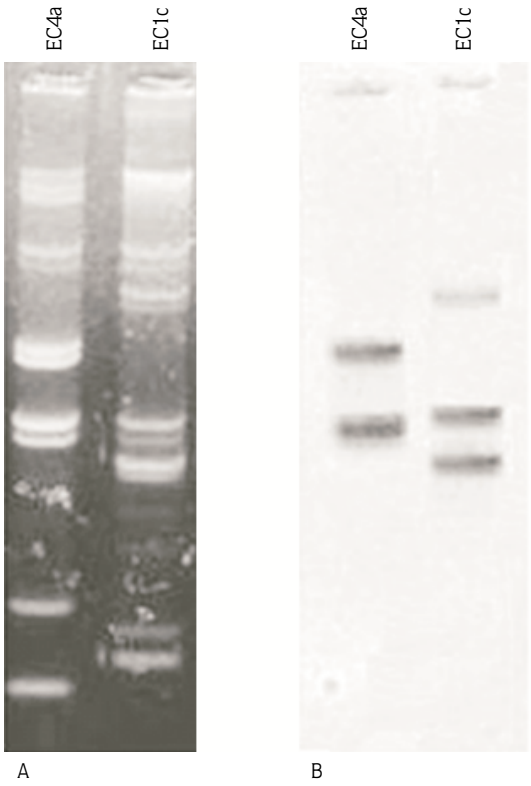

Figure 3. Agarose gel electrophoresis of plasmid DNAs from the ampicillin-resistant *E. coli* strains EC1c and EC4a (A); Plasmid DNAs from the ampicillin-resistant *E. coli* strains EC1c and EC4a hybridized with digoxigenin-labeled TEM-1 probe (B).

R plasmids; however, 10 Amp^r *E. coli* strains were found to be carried by the volunteers (Table 1). This prevalence rate is higher than those reported in previous studies by Degener et al. (23) and Bonten et al. (24) performed in developed countries, namely 6% and 8%, respectively. On the one hand, Shears et al. (25) and Okeke et al. (26) reported prevalence rates among children in the Sudan and Nigeria as 96% and 35%, respectively. On the other, Gülay et al. (27) reported a higher prevalence rate (78%) than our result from Turkey. They collected fecal specimens from 50 healthy volunteers living in İzmir, a large populated city on the Aegean Sea coastline in western Turkey, and examined them for the presence of β-lactamase producing *E. coli* strains. Although the exact molecular mechanisms could not be determined, their study showed that the incidence of ampicillin resistance was also high in commensal fecal flora.

Ampicillin resistance in normal flora is found to be due mainly to the independent acquisition of the TEM-1 gene by different plasmids rather than to the presence of epidemic strains or plasmids (28). Our plasmid analyses showed that plasmid DNA bands ranged from 0.5 to >10 kb in ampicillin-resistant isolates, and four of 10 *E. coli* strains were found to be plasmidless (Table 1 and Figure 1). There seems to be no association between the presence of large plasmid DNA and ampicillin resistance because it is apparent in the light of current experiments that some plasmid DNAs in the isolates were detected not to carry any *bla* genes by PCR. We are of the opinion that they probably carry cryptic features such as virulence, metabolic or adhesion factors excluding the antibiotic resistance determinants. However, in other studies (25,29) performed in developing countries, it was found that the conjugative plasmid sizes ranging from 42 to 80 MDa were associated with different resistance patterns in *Enterobacteriaceae* isolated from fecal flora.

Two of R$^+$ transconjugants (R$^+$ [*E. coli* EC1c] and R$^+$ [*E. coli* EC4a]) harboring bla_{TEM} genes were resistant to ampicillin, ampicillin/sulbactam and cefazolin, but sensitive to ceftazidime. It has been known that some of OXA- and SHV-derived β-lactamases can also hydrolyze

the inhibitor combinations other than ampicillin (12). Nevertheless, bla_{SHV} or bla_{OXA} genes could not be detected in representative strains and their transconjugants. According to these results, it might be suggested that the strains are hyperproducers of TEM-1 or TEM-2 penicillinases. Overproduction of TEM-1 penicillinase in *E. coli* results in increased resistance to a wide range of β-lactams and β-lactam/β-lactamase inhibitor combinations (30). In the current study, we detected that these hyperproducers resisted to ampicillin at a high concentration, with MIC of >512 µg/mL. Pitout et al. (31) reported that prevalence of resistance to ampicillin and older cephalosporins is on the increase in *E. coli* due primarily to the dissemination of plasmids encoding TEM-1 or SHV-1 β-lactamases.

TEM-type of β-lactamase genes show a remarkable tendency to mutate to give enzymes with an extended-spectrum of activity (30). These mutants are generally present in hospitalized patients, but they will be carried out of the environment due to the discharge of their carriers, adding to the load of resistance genes in the wider community. Furthermore, Balis et al. (32) also reported the indications of *in vivo* transfer of an epidemic R plasmid between ampicillin-resistant *Salmonella enteritidis* and *E. coli* in normal human gut flora, and suggested that these organisms had the possible role of normal flora as a reservoir of resistance genes. On the other hand, Heritage et al. (33) found that 24% of fecal flora in general practice patients contained ampicillin-resistant cultivable and non-cultivable bacteria by PCR-based method, and also suggested that this was important for detecting antibiotic resistance genes to identify persons in the community who harbor bowel flora in which bla_{TEM} genes were present.

In this study, results of the hybridization assays (Figure 3B) imply that there are multiple plasmids carrying the bla_{TEM} gene within single Amp^r *E. coli* strains, but both of two strains carry single plasmid bearing bla_{TEM} gene in reality; the leading is the supercoiled plasmid, and the remaining is open circles or multimer (probably dimmer) formations of the same plasmid, which resulted from the rough preparation. Hence, we decided that the actual sizes of these small mobilizable plasmids in the strains were 2.5 kb and 3 kb in sizes, respectively, as seen in Figure 1 and Figure 3A. However, looking at Figure 1 showing agarose gel electrophoresis of the plasmids isolated from the original strains EC1c and EC4a, the large plasmids which located between wells and chromosomal DNA (Chr. DNA) can be seen in lane 3 and lane 6, but no hybridization of TEM-1 probe to these large plasmids was detected (Figure 3B). We think that these small plasmids could have been mobilized by another self-transmissible cryptic plasmid encoding unknown characteristics and with low copy number (1 to 2 copy/chromosome) because they are by far too small to be themselves conjugative. The findings presented here suggest that bla_{TEM} genes in commensal *E. coli* strains colonizing to the normal gut environment are maintained by means of small mobilizable R plasmids which are no burden to the bacterial metabolism. The authors in the current study also encountered this kind of small mobilizable R plasmid even in *E. coli* strains with clinical origin in another study (34).

Plasmids of the *E. coli* incompatibility group Q (IncQ) and related IncQ-like plasmids are characterized by their ability to be mobilized by several self-transmissible plasmids, their relatively small size, and their broad host range (35). It would be useful in future related studies for structures and regulation mechanisms of R plasmid-bearing-genes for TEM-β-lactamases, mobilization and transfer complex to be characterized and clarified by further molecular techniques.

We believe that screening for the presence of such small mobilizable R plasmids and enlightenment of the dissemination mechanisms will generate a useful and meaningful database as to the availability of potential resistance genes in the community. The data here show that commensal *E. coli* strains bearing TEM-genes can easily emerge and survive in the bowel environment of healthy persons ''even in the absence of recent antibiotic consumption'', and these resistance determinants are retained by small extrachromosomal DNA elements in bacteria belonging to the microflora of the intestinal tract.

The TEM-1 type of penicillinase gene is commonly carried by about 4.95 kb-Tn*3* transposon, highly widespread on a wide range of incompatible plasmids, commonly emerging in the *Enterobacteriaceae* family (36). Interestingly, the TEM-gene bearing R plasmids in this study are too small to carry Tn*3* transposon. Thus, we consider that such R plasmids probably carry and disseminate bla_{TEM} genes by a partial transposon. In this respect, we suggest that sequencing of such small R plasmids completely or at least the part carrying the

resistance genes and their adjacent regions would supply basic information to other studies, and may be of relevance with regard to the further dissemination of these bla_{TEM} genes, prompting examination of the evolution of antibiotic resistance in the community.

Dissemination of the resistance genes between organisms in fecal flora illustrates the public health importance. To detect the reservoirs of potential resistance genes in the community, and to understand their maintenance mechanisms, fecal flora of healthy persons should be regularly screened and investigated for the carriage of the given antibiotic resistance genes because the actual risk to public health is the transfer of resistance genes from the commensal bacteria to human pathogens.

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