Investigation of diversity of plasmids carrying the $bla_{TEM-52}$ gene

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Objectives: To investigate the diversity of plasmids that carry $bla_{TEM-52}$ genes among Escherichia coli and Salmonella enterica originating from animals, meat products and humans.

Methods: A collection of 22 $bla_{TEM-52}$-encoding plasmids was characterized by restriction fragment length polymorphism (RFLP), replicon typing (by PCR or replicon sequencing), susceptibility testing, assessment of plasmid ability to self-transfer by conjugation and typing of the genetic environment of the $bla_{TEM-52}$ gene. Detected IncI1 plasmids underwent further plasmid multilocus sequence typing.

Results: RFLP profiles demonstrated dissemination of $bla_{TEM-52}$ in Denmark (imported meat from Germany), France, Belgium and the Netherlands from 2000 to 2006 by mainly two different plasmids, one encoding $bla_{TEM-52a}$ (IncX1A, 45 kb) and the other $bla_{TEM-52c}$ (IncI1, 80 kb). In addition, $bla_{TEM-52b}$ was also found to be located on various other plasmids belonging to IncA/C and IncL/M, while $bla_{TEM-52e}$ was found on IncN-like as well as on IncR plasmids. In the majority of cases (n = 21) the $bla_{TEM-52}$ gene was located on a Tn3 transposon. Seven out of 10 $bla_{TEM-52}$ plasmids tested in conjugation experiments were shown to be capable of self-transfer to a plasmid-free E. coli recipient.

Conclusions: The $bla_{TEM-52}$ gene found in humans could have been transmitted on transferable plasmids originating from animal sources. Some of the $bla_{TEM-52}$ plasmids carry replicons that differ from the classical ones. Two novel replicons were detected, IncX1A and IncN-like. Unlike its predecessor $bla_{TEM-1}$, the $bla_{TEM-52}$ gene was not detected on F-type replicons suggesting that this gene evolved on other types of plasmid scaffolds.

Keywords: antibiotic resistance, ESBLs, human and non-human isolates

Introduction

Plasmids are usually circular, double-stranded DNA entities that can self-replicate. They often encode functions giving extra advantages to their hosts in the presence of selective pressure. The ability of some plasmids to transfer antibiotic resistance genes from one bacterial host to another constitutes a real threat to human health. Extended-spectrum β-lactam antibiotics are commonly used for treatment of severe infections caused by Gram-negative bacteria, in particular those caused by Enterobacteriaceae. Resistance to these β-lactams is often plasmid encoded and the frequency of resistance is alarmingly increasing.

Plasmid-encoded BlaTEM-type enzymes capable of degrading β-lactam antibiotics were first described in 1965 and have since then disseminated worldwide. The $bla_{TEM-52}$ Gene, encoding an extended-spectrum β-lactamase (ESBL), was first described in 1998 in France. Since then it has been detected in clinical isolates and/or production animals from Canada, Portugal, France, Greece, the Netherlands, Germany, Belgium, Great Britain, Croatia and Japan, and has become the most prevalent ESBL in Korea, where it seemed to spread both clonally and horizontally. Currently $bla_{TEM-52}$ β-lactamases constitute one of the most common types of ESBLs along with the $bla_{SHV}$ and $bla_{CTX-M}$ enzymes. Many of the reports on the occurrence of $bla_{TEM-52}$ genes come from ESBL prevalence studies, which did not focus on detailed characterization of plasmid species associated with the $bla_{TEM-52}$ genes. Thus little is known about the possible relationship between plasmids encoding the $bla_{TEM-52}$-type β-lactamases. Knowledge about the mechanisms of dissemination of β-lactam resistance traits by mobile elements like plasmids can facilitate development of methods for predicting and further controlling that dissemination.

Currently, the largest amount of data on plasmids harboursing ESBL genes exists for plasmids carrying $bla_{CTX-M}$ genes. Replicons belonging to IncI1, IncN, IncFIB, IncFIA, IncFI1, IncA/C, IncL/M and IncH12 families were, in the majority of cases, associated with diverse subtypes of $bla_{CTX-M}$. Overall, other β-lactamases are readily transferred from one host to another as a consequence of horizontal gene transfer, including conjugation, transduction, and transformation.
resistance genes like bla<sub>TEM</sub>-52, bla<sub>TEM</sub>-52a, bla<sub>TEM</sub>-52b, and diverse bla<sub>TEM</sub> subtypes were also most often localized on plasmids carrying the aforementioned replicons. 1 Replicons belonging to other Inc families were detected sporadically on the ESBLs encoding plasmids. 1

Different bla<sub>TEM</sub><sub>–</sub>52<sub>–</sub> genes, including bla<sub>TEM</sub>-52<sub>–</sub>2, evolved from bla<sub>TEM</sub>-52<sub>–</sub>1 and bla<sub>TEM</sub>-52<sub>–</sub>2. 2 Bla<sub>TEM</sub>-52<sub>–</sub>1 and Bla<sub>TEM</sub>-52<sub>–</sub>2 β-lactamases are not considered ESBLs due to their narrow substrate spectrum. Subsequent mutations in the bla<sub>TEM</sub>-52<sub>–</sub>1 genes led to amino acid substitutions that expanded the substrate spectrum of the encoded enzyme due to an enlargement of the active site. 2 Bla<sub>TEM</sub>-52<sub>–</sub>2 differs from the Bla<sub>TEM</sub>-52<sub>–</sub>1 β-lactamase by three amino acid substitutions; Glu(104) → Lys, Met(182) → Thr and Gly(238) → Ser. 18 Also the silent point mutations are useful in tracing the evolutionary origin of the resistance genes. 18 Thus far two variants of the bla<sub>TEM</sub>-52<sub>–</sub>2 gene (bla<sub>TEM</sub>-52<sub>–</sub>2a and bla<sub>TEM</sub>-52<sub>–</sub>2b) have been described. 4–7,9–11,19 Detection of similar plasmids harbouring different alleles of the bla<sub>TEM</sub> genes would indicate that the plasmids might have acquired these genes possibly on transposable elements from different sources. 6

The sparse knowledge about the possible relationship between plasmids harbouring the bla<sub>TEM</sub>-52<sub>–</sub> resistance genes prompted us to conduct a study on these plasmids in order to further insight into their dissemination among the Enterobacteriaceae. Plasmids from both human and animal (or meat) isolates were analysed to investigate a potential plasmid-associated transfer of bla<sub>TEM</sub>-52<sub>–</sub> from animal to human reservoirs.

Materials and methods
Selection of strains
Twenty-two strains including Escherichia coli (n = 13) and various serovars of Salmonella enterica (n = 9) and carrying a version of the bla<sub>TEM</sub>-52<sub>–</sub> gene were collected from different sources and further characterized in this study (see Table 1). Isolates were collected during the period from 1995 to 2006 in different countries (Denmark (German meat), France, the Netherlands, Belgium, Spain, Korea and Canada). They were kindly provided by different researchers and institutes and originated from poultry, poultry meat, beef meat or clinical samples from humans. 5–7,9–11,19

Isolation of individual plasmids carrying the bla<sub>TEM</sub>-52<sub>–</sub> gene
Plasmidic DNA was purified from wild-type isolates and later from transformants using a Qiagen Plasmid Mini or Midi Kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. Plasmids were introduced to electrocompetent plasmid-free E. coli GeneHogs® (Invitrogen) cells by means of transformation by electroporation (Bio-Rad, Micropulser™). The transformation set-up was as follows: 2.5 kV; resistance 200 Ω, and capacitance 25 μF. Electroporants (transformants) were resuspended in 1.2 mL of brain hearth infusion (BHI) broth (Becton, Dickinson & Co., Bacto™), incubated for a minimum of 1 h at 37°C and plated onto selective BHI (Becton, Dickinson & Co., Difco™) agar plates.

Verification of transformants harbouring individual plasmids carrying the bla<sub>TEM</sub>-52<sub>–</sub> gene
Plasmids from the 22 donor strains were used for transformation by electroporation as described above. Selection of transformants (further designated with the suffix TF) was done on agar plates containing 2 mg/L cefotaxime. If necessary, plasmids from transformants were purified as described previously and the procedure was repeated until transformants with single bla<sub>TEM</sub>-52<sub>–</sub> plasmids were isolated for all 22 corresponding primary strains.

The presence of plasmids in the transformants and their sizes were determined using S1-PFGE; 20 5 U of S1 nuclelease (Fermentas) was used per plug slice. Plug slices with Xbal-digested Salmonella Brandenup were used as size ladders. 21 Samples were run on a CHEF-DR III System (Bio-Rad Laboratories, Hercules, CA, USA) and the conditions used were a 1% agarose gel (SeaKem Gold Agarose/Lonza) in 0.5x Tris/borate/EDTA, a voltage gradient of 6 V/cm, with phase from 6.8 to 38.4 s, and a run time of 19 h. Staining and image capture were performed as stated in Ribot et al. 21

Verification of bla<sub>TEM</sub>-52<sub>–</sub> genes
The presence of ESBLs genes was confirmed for both donor strains and transformants by PCR targeting conserved regions flanking the bla<sub>TEM</sub> gene, as described by Hasman et al. 9 Unless the bla<sub>TEM</sub>-52<sub>–</sub> gene was already sequenced, PCR products were purified using GFX columns (Amersham Biosciences) and fully sequenced (Macrogen Inc., Korea).

Plasmid characterization by restriction fragment length polymorphism (RFLP)
RFLP was performed on bla<sub>TEM</sub>-52<sub>–</sub> plasmids from the 22 transformants. Plasmids from all transformants were purified as described and digested with EcoRI. The resulting fragments were separated and visualized on a 0.8% agarose gel (SeaKem Gold Agarose/Lonza) after 5 h at 4.0 V/cm or 21 h at 1.2 V/cm.

Replicon typing
Replicons of plasmids from the transformants were typed as described by Carattoli and co-workers. 22,23 In cases when it was impossible to determine the replicon by this method, cloning of the replicon or full plasmid sequencing was performed using a GS FLX pyrosequencer (Roche).

From plasmid preparations of pE001 (located in E. coli 2161TF) and pGOC049 (located in E. coli GOC09TF), a standard FLX sequencing library was built using 5 μg of DNA according to the manufacturer’s guidelines (Roche). Test emulsion PCRs were performed to obtain the best copies/bead ratio. DNA containing beads with each plasmid library were sequenced in two regions using the GS FLX standard sequencing kit on a 4-region 25×75 pico titre plate. A total of 11 000 and 20 000 reads from each of plasmids pE001 and pGOC049 were aligned and assembled using the Newbler assembler software version 2.0.01.14 provided with the GS FLX instrument.

In the case of the plasmid from 727TF, a fragment carrying the putative replicon was generated by digestion of the plasmid with BglI and BamHI (Fermentas) and subsequent purification on the GFX column (Amersham Biosciences). The fragment was ligated to the chloramphenicol resistance gene that was PCR amplified from the vector pLOW1 (Amersham Life Science). The construct was transformed by electroporation into electrocompetent GeneHogs®. Transformant was selected on BHI agar plates supplied with 25 mg/L chloramphenicol. Plasmid was purified from this transformant and used as the template for sequencing. Sequencing was performed by the standard Sanger sequencing method at Macrogen Inc. (Korea).

Results were further processed using Vector NTI Suite 11 (Invitrogen, Inc.) and then BLASTN and BLASTX searches against known replicon sequences from the GenBank database were performed to identify putative replication proteins located on the plasmids.
Investigation of diversity of plasmids carrying the bla_{TEM-52} gene

Multilocus sequence typing of IncI1 plasmids

All plasmids from transformants positive for the IncI1 replicon in the multiplex PCR were found to carry the IncI1 replicon. PCR linking for the presence of the IncI1 replicon in the transformants was confirmed by PCR targeting replicons of R46 and 96, respectively. Each of the four transformants mentioned in this section was made electrocompetent using a standard protocol for preparation of electroporated E. coli cells. The protocol employed to perform the incompatibility assay was as described by Norman et al. with modification so that the tested plasmid was introduced into the electroporated cells harbouring the second plasmid by means of transformation by electroporation. pGOC049 (CTXr) was tested against IncN representative plasmid R46 (Tet). Transformation of these strains with both pGOC049 and R46 was made on an agar plate supplied with cefotaxime together with tetracycline (2 mg/L and 16 mg/L, respectively). pE001 (CTXr) was tested against IncI1 representative pOAL52 bla::npt (Kan). An agar plate supplied with cefotaxime together with kanamycin (2 mg/L and 50 mg/L, respectively) was used to select transformants harbouring both pOAL52 bla::npt and pE001. Each of the two tested plasmids was used in the incompatibility assay both as the incoming and as the resident agent. To assess the plasmid losses in the incompatibility assay for pGOCO49 and R46, selective plates with 16 mg/L tetracycline and 2 mg/L cefotaxime were used, respectively. To assess plasmid losses in the incompatibility assay for pE001 against pOAL52 bla::npt, selective plates supplied with 2 mg/L cefotaxime and 50 mg/L kanamycin were used, respectively. As controls, each of the four transformants originally harbouring only one plasmid was inoculated into the broth without selection, and plasmid stability was further assessed following the method described by Norman et al. The presence/absence of specific replicon types in the obtained transformants was confirmed by PCR targeting replicons of R46, pOAL52 bla::npt, pE001 and pGOC049, respectively.

PCR conditions for detecting IncN replicon of R46 were as described by Carattoli et al. Primers for detecting the remaining replicons were designed based on the obtained sequencing data. For the sequences of the primers and for PCR details please see Table S1 (available as Supplementary data at JAC Online).

Incompatibility assay

Incompatibility testing was performed for bla_{TEM-52} plasmids located in E. coli GOC049TF (designated as pGOC049) and E. coli 2161TF (designated as pE001). In separate transformations, E. coli GeneHogs carrying plasmid R46 (IncN plasmid, kindly provided by Alessandra Carattoli, Istituto Superiore di Sanità, Rome, Italy; accession number AY046276) and the IncX1 plasmid pOAL52 bla::npt (Kan), respectively, were obtained. Plasmids were purified from these by the described method. Each of the four transformants mentioned in this section was made electrocompetent using a standard protocol for preparation of electrically competent E. coli cells. The protocol employed to perform the incompatibility assay was as described by Norman et al. with modification so that the tested plasmid was introduced into the electroporated cells harbouring the second plasmid by means of transformation by electroporation. pGOC049 (CTXr) was tested against IncN representative plasmid R46 (Tet). Transformation of these strains with both pGOC049 and R46 was made on an agar plate supplied with cefotaxime together with tetracycline (2 mg/L and 16 mg/L, respectively). pE001 (CTXr) was tested against IncI1 representative pOAL52 bla::npt (Kan). An agar plate supplied with cefotaxime together with kanamycin (2 mg/L and 50 mg/L, respectively) was used to select transformants harbouring both pOAL52 bla::npt and pE001. Each of the two tested plasmids was used in the incompatibility assay both as the incoming and as the resident agent. To assess the plasmid losses in the incompatibility assay for pGOC049 and R46, selective plates with 16 mg/L tetracycline and 2 mg/L cefotaxime were used, respectively. To assess plasmid losses in the incompatibility assay for pE001 against pOAL52 bla::npt, selective plates supplied with 2 mg/L cefotaxime and 50 mg/L kanamycin were used, respectively. As controls, each of the four transformants originally harbouring only one plasmid was inoculated into the broth without selection, and plasmid stability was further assessed following the method described by Norman et al. The presence/absence of specific replicon types in the obtained transformants was confirmed by PCR targeting replicons of R46, pOAL52 bla::npt, pE001 and pGOC049, respectively.

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Examination of clonal relationship of wild-type isolates harbouring similar plasmids

If plasmids carrying the same replicons and displaying similar RFLP profiles were detected in more than one of the transformants, the corresponding E. coli wild-type strains harbouring similar plasmids were tested by a PCR phylotyping method as described by Clermont et al. to pre-determine the potential clonality of these strains.

Genetic environment upstream of the bla_{TEM-52} gene

PCR linking for the presence of the InpA gene of Tn3 upstream of the bla_{TEM-52} gene was performed on the transformants (n = 22). The primers used and PCR details are given in Table S1. The PCR product obtained from strain 54.12TF was purified using GFX columns (Amersham Biosciences) and sequenced. The sequence was aligned with the sequence of Tn3-bla_{TEM-52} (EF141186).

Plasmid transmissibility

bla_{TEM-52}-Carrying plasmids from selected strains (representing each of the different RFLP groups {76-33094TF, 44.02TF, ESBL 140TF, ESBL 424TF, YMC 95/4/4199TF, YMC 96/7/4035TF, 549TF, 641TF, GOC043TF and 727TF}) were tested for the ability to self-transfer to the plasmid-free recipient E. coli MT101 (Naïf, Rif²). Conjugation was set-up as follows: sterile paper filter (pore diameter 0.2 μm, Advantec) was placed in the centre of a blood agar plate, 2 mL of donor and recipient cultures in exponential phases of growth were mixed together and 500 μL of the mixture was placed on the paper filter, allowing the liquid to soak into the medium. After overnight incubation, filters were washed with 4 mL of 0.9% salt water and 100 μL of the suspension was inoculated onto BHI (Becton, Dickinson & Co., Difco™) agar plates with 2 mg/L cefotaxime, 32 mg/L nalidixic acid and 25 mg/L rifampicin. After overnight incubation at 37°C the presence of transconjugants was assessed.

Susceptibility testing

Unless stated in the references, the primary strains were tested for their susceptibility to a range of antimicrobial agents by means of a commercially available panel for Enterobacteriaceae (Sensititre). The antimicrobial agents included were amoxicillin/clavulanic acid, ampicillin, apramycin, cefotaxime, ceftiofur, chloramphenicol, ciprofloxacin, colistin, florfenicol, gentamicin, nalidixic acid, neomycin, spectinomycin, streptomycin, sulfamethoxazole, tetracycline and trimethoprim. Testing was performed according to the recommendations of the CLSI (formerly the NCCLS). CLSI breakpoints (2003) were used for interpretation of the results except for cefotaxime. For ceftiofur, and if no CLSI breakpoints were available for a tested compound, the criteria of the European Committee on Antimicrobial Susceptibility Testing (EUCAST) were used for interpretation of the results.

Based on the resistance profiles obtained for donor strains, transformants were tested for susceptibility to the selected antimicrobials. Susceptibility testing of transformants was performed using the disc diffusion method according to the guidelines of the CLSI. The recipient strain of E. coli GeneHogs was included as a control. CLSI zone diameter interpretative standards (2003) were used for interpretation of the results. Results for ceftiofur were interpreted as recommended by Aarestrup et al.

Results

Verification of bla_{TEM-52} genes

Twenty-two strains carrying a bla_{TEM-52} plasmid were obtained from different sources as listed in Table 1 and transferred to a plasmid-free E. coli recipient. The obtained transformants were given the same names as the corresponding donor isolates, but with a T suffix. Both the primary strains and the obtained transformants were positive in PCR targeting the bla_{TEM-52} gene. Among these isolates, 13 were found to carry a plasmid with the bla_{TEM-52}b version of the gene and 9 carried the bla_{TEM-52}c version.

Plasmid characterization by RFLP

Purified plasmids from all 22 transformant strains were digested with the EcoRI enzyme. RFLP patterns indicated that strains 2161TF, 7633094-7TF, 36.52TF, 44.02TF, 46.20TF, 48.78TF,
Table 1. List of wild-type strains harbouring the bla\textsubscript{TEM-52} plasmids characterized in the study and the results of plasmid characterization

<table>
<thead>
<tr>
<th>Wild-type strain</th>
<th>Phyotype</th>
<th>Isolation source (country of origin)</th>
<th>Year of isolation</th>
<th>bla\textsubscript{TEM-52} allele</th>
<th>Plasmid RFLP type</th>
<th>Replicon type</th>
<th>Self-transmissibility</th>
<th>Plasmid size (kb)</th>
<th>Element upstream of bla\textsubscript{TEM-52}</th>
<th>Resistances associated with the bla\textsubscript{TEM-52} plasmid</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli 2161</td>
<td>B1</td>
<td>broiler meat (DE)</td>
<td>2006</td>
<td>TEM-S2b\textsuperscript{b}</td>
<td>a IncX1A</td>
<td>ND</td>
<td>yes</td>
<td>45</td>
<td>Tn3</td>
<td>AMP, CEF, CPO, XNL</td>
<td>4</td>
</tr>
<tr>
<td>E. coli 7633094-7</td>
<td>B1</td>
<td>beef (DE)</td>
<td>2004</td>
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<td>AMP, CEF, XNL</td>
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<td>poultry meat (NL)</td>
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<td>ND</td>
<td>yes</td>
<td>45</td>
<td>Tn3</td>
<td>AMP, CEF, XNL</td>
<td>9</td>
</tr>
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<td></td>
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<tr>
<td>Salmonella Blockley</td>
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<td>ND</td>
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<td>2002</td>
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<td>human (F)</td>
<td>2002</td>
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<td>Tn3</td>
<td>AMP, XNL, [CTX]</td>
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<td>e IncA/C</td>
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<td>Tn3</td>
<td>AMP, XNL, CTX, GEN, SPT, TET</td>
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<tr>
<td>Salmonella Saintpaul YMC 95/4/4199</td>
<td></td>
<td>human (KR)</td>
<td>1995–97</td>
<td>TEM-S2b\textsuperscript{g}</td>
<td>IncL/M</td>
<td>yes</td>
<td>65</td>
<td>Tn3</td>
<td>AMP, XNL, CTX, SPT, SXT, SMX, TMP</td>
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<tr>
<td>Salmonella Stanley YMC 96/7/4035</td>
<td></td>
<td>human (KR)</td>
<td>1995–97</td>
<td>TEM-S2b\textsuperscript{h}</td>
<td>IncL/M</td>
<td>yes</td>
<td>80</td>
<td>Tn3</td>
<td>AMP, XNL, CTX, GEN</td>
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DE, Denmark; NL, the Netherlands; F, France; B, Belgium; ES, Spain; CA, Canada; KR, Korea; ND, not determined; NA, not applicable; AMP, ampicillin; AMC, amoxicillin/clavulanate (2:1); CAZ, ceftazidime; CEF, cefalotin; CPO, cepodoxime; CRO, ceftriaxone; CTX, cefotaxime; GEN, gentamicin; NEO, neomycin; SMX, sulfamethoxazole; TET, tetracycline; TMP, trimethoprim; XNL, ceftiofur; SPT, spectinomycin; [], intermediate resistance.

\textsuperscript{a}Gene was sequenced by the researcher/institute providing the strain.

\textsuperscript{b}Isolated in Denmark.

\textsuperscript{c}Plasmid originally isolated from \textit{S. enterica}; transconjugant provided by Cloeckaert et al.\textsuperscript{7}
51.09TF, 54.12TF and 660TF shared a very similar bla<sub>TEM-52</sub>-type plasmid with an approximate size of 45 kb. The RFLP profile of these plasmids was designated type a. Strains 44.78TF, 549TF, 641TF, 692TF, 710TF and Cloeckaert TF were shown to share a similar ~80 kb bla<sub>TEM-52</sub>-type plasmid, which differed from the already mentioned pBI<sub>TEM-52</sub>, RFLP type a. This RFLP type was designated type b, pGOC043 and pGOC049 shared an almost identical pattern, as they differed only by the presence of one extra band in pGOC049, and were thus designated as type c. RFLP profiles of plasmids from the remaining transformants were significantly different from each other (difference of more than six bands) and from the three described types. These plasmids ranged in size from ~40 kb to ~146 kb and were designated with RFLP profile letters d to h.

**Replicon typing**

All transformants carrying plasmids with RFLP profile b and ESBL 424TF (RFLP type f) were positive for the IncI1 replicon in the multiplex PCR. YMC 95/4/4199TF (RFLP type g) and YMC 96/7/424TF (RFLP type h) were positive for the IncL/M replicon. In one case for ESBL 140TF (RFLP type e) the replicon InAC was detected and 727TF (type d) gave a positive signal for the IncR replicon.

Replicons of plasmids from 2161TF (representing type a) and GOC49TF (representing type c) did not produce positive results by the standard multiplex PCR and were therefore detected by partial or full plasmid sequencing. As the IncR replicon is not so commonly detected in *E. coli*, the replicon of plasmid from 727TF (plasmid designated as p727) was also cloned and sequenced to perform further analysis. The sequences were compared with the GenBank database to identify similar plasmids with known replications. The sequence encoding the putative replicase of plasmid p727 showed 100% similarity with the repA sequence of plasmid p727. Regardless of which of the plasmids was the incoming or residing agent in the assay, in both cases 99% of transformants by the PCRs targeting these replicons. This demonstrated the stability of the plasmids in the recipient. The pE001 plasmid turned out to be compatible with pOLA52 bla::npt (an IncX1 plasmid). Regardless of which of the plasmids was the incoming or residing agent in the assay, in both cases 99% of transformants retained the initial resistance to both cephalothin and kanamycin, indicating that the two plasmids could be present in the same cell. Two selected transformants with resistance to both antibiotics underwent S1-PFGE, and they were shown to harbour two plasmids at the same time, thus demonstrating that no co-integration had occurred. This led to the conclusion that the two plasmids are compatible. However, based on the large homology of the overall pE001 sequence with pOLA52 and 100% identity of the putative rep of pE001 with pir of pMAS2027 (classified as IncX1<sup>16</sup>), we suggest assigning this plasmid to the IncX family, and further to the IncX1A subgroup.

**Multilocus sequence typing of IncI1 plasmids**

Plasmids positive for the IncI1 replicon in the multiplex PCR, namely from ESBL 424TF, 44.78TF, 549TF, 641TF, 692TF, 710TF and Cloeckaert TF, underwent further pMLST<sup>22,24</sup> Based on the sequencing results, the plasmid from ESBL 424TF (RFLP type f) was assigned to be of sequence type (ST) 2. The remaining tested plasmids (sharing RFLP type b) were assigned by pMLST to ST5. Various insertions or deletions were observed in the sequences obtained for the six IncI1 plasmids of ST5 compared with the allele variants described in the reference. However, these mutations were not located on the sites corresponding to the relevant nucleotides, e.g. those determining the ST5 on the reference sequences.

**Incompatibility testing**

Incompatibility testing was performed for bla<sub>TEM-52</sub> plasmids originating from *E. coli* GOC049TF (pGOC049) and *E. coli* 2161TF (pE001) to investigate their incompatibility affiliation. In transformants harbouring only one of the respective plasmids, e.g. pGOC049, R46, pOLA52 bla::npt or pE001, and grown for 50 generations without selective pressure, no loss of original resistance was observed. The presence of respective replicons was additionally confirmed in selected colonies from the transformants by the PCRs targeting these replicons. This demonstrated the stability of the plasmids in the recipient. The pE001 plasmid turned out to be compatible with pOLA52 bla::npt (an IncX1 plasmid). Regardless of which of the plasmids was the incoming or residing agent in the assay, in both cases 99% of transformants retained the initial resistance to both cephalothin and kanamycin, indicating that the two plasmids could co-exist in the same cell. Two selected transformants with resistance to both antibiotics underwent S1-PFGE, and they were shown to harbour two plasmids at the same time, thus demonstrating that no co-integration had occurred. This led to the conclusion that the two plasmids are compatible. However, based on the large homology of the overall pE001 sequence with pOLA52 and 100% identity of the putative rep of pE001 with pir of pMAS2027 (classified as IncX1<sup>16</sup>), we suggest assigning this plasmid to the IncX family, and further to the IncX1A subgroup.

**Replication of pE001**

The rep sequence of pGOC049 showed 72% identity with the repA sequence of the classical IncN plasmid R46. Attempts to introduce pGOC049 into electrocompetent cells already...
harbouring R46 did not produce transformants on the plate with selection for both plasmids at the same time. However, transformants were observed on this selective plate when R46 was used as the incoming agent and pGOC049 as the residing one. What is more, no loss of resistance either to cefotaxime or to tetracycline was observed after cultivating that transformant for 50 generations without selection. At the end of the incompatibility assay a selected transformant colony that was resistant to cefotaxime or to tetracycline was observed.
both cefotaxime and tetracycline was tested using S1-PFGE and was shown to harbour the two plasmids at the same time. The results obtained from the incompatibility assay are difficult to interpret since transformants harbouring two plasmids simultaneously were obtained only when R46 was the incoming plasmid, but not when the pGOC049 was the incoming agent. Due to the similarity of the two replicons we propose to term the replicon of pGOC049 as IncN-like.

**Examination of clonal relationship of wild-type isolates harbouring similar plasmids**

Three *E. coli* strains 2161, 7633094-7 and 660 harboured similar IncX1A plasmids. *E. coli* 2161 and 7633094-7 were shown to belong to the same phylotype B1, and thus could possibly be clonally related. *E. coli* 660 was assigned to phylotype A and would not be expected to be clonally related to the two phylotype B1 isolates with similar plasmids. *E. coli* 549, 641, 692 and 710 strains harboured very similar IncI1 plasmids. Two of these isolates, *E. coli* 549 and 692, shared the same phylotype A and could be clonally related. *E. coli* 641 and 710 were shown to belong to B1 and D phylotypes, respectively. *E. coli* GOC043 and GOC049 harboured similar IncN-like plasmids. These two strains gave a positive signal in the PCR to B2 and B1 phylotypes, respectively.

The Salmonella Blokley wild-type isolates 36.52, 46.20 and 51.09 harbouring similar IncX1A plasmids have been previously examined by XbaI-digested PFGE by Hasman and Aarestrup.3

**Genetic environment upstream of the bla**$_{TEM-52}$**gene**

In the plasmid originating from the Cloeckaert TK isolate the bla$_{TEM-52c}$ gene had previously been reported to reside on a Tn3 element.7 Thus the upstream regions of bla$_{TEM-52}$ genes in plasmids from the remaining transformants of this study were also investigated. For the plasmid originating from ESBL 140TF (RFLP type e), no signal was observed in the PCR linking the presence of tnpA with the bla$_{TEM-52}$ gene. The remaining plasmids were positive in the described PCR, indicating that the bla$_{TEM-52}$ genes were located within the Tn3 transposon and downstream of the transposese.

**Plasmid transmissibility**

bla$_{TEM-52}$ plasmids belonging to different RFLP groups (indicated in parentheses) and originating from selected transformants that were shown not to carry any other plasmids—76-33094TF (RFLP type a), 44.02TF (RFLP type a), ESBL 140TF (RFLP type e), ESBL 424TF (RFLP type f), YMC 95/4/4199TF (RFLP type g), YMC 96/7/4035TF (RFLP type h), 549TF (RFLP type b), 641TF (RFLP type b), GOC043TF (RFLP type c) and 727TF (RFLP type d)—were tested for the ability to self-transfer to the plasmid-free recipient *E. coli* MT101. Transconjugants were observed for all the above listed strains except three; 549TF (RFLP type b/IncI1), ESBL 140TF (RFLP type e/IncA/C) and 727TF (RFLP type d/IncR).

**Susceptibility testing**

Not surprisingly, all primary strains were resistant to ampicillin, ceftiofur and cefotaxime. All 22 transformants that carried only a variant of a bla$_{TEM-52}$ plasmid were likewise resistant to the tested β-lactam antimicrobials.

Thirteen of the primary strains were resistant to sulphonamides and trimethoprim. In one case these resistances were apparently associated with the bla$_{TEM-52b}$/IncL/M plasmid from YMC 95/4/4199TF. Ten primary strains were resistant to tetracycline. Tetracycline resistance associated with the bla$_{TEM-52b}$/IncI1 plasmid was observed in one case in the ESBL 424TF strain. Five of the primary strains were resistant to neomycin. This resistance was observed in one of the corresponding transformants, namely 727TF, indicating that it was residing on the IncR bla$_{TEM-52c}$/plasmid. Five of the primary isolates were resistant to gentamicin and in four cases this resistance was associated with bla$_{TEM-52b}$/ IncR plasmids from ESBL 140TF (IncA/C plasmid), ESBL 424TF (IncI1), 727TF (IncR) and YMC 96/7/4035TF (IncL/M). Five of the primary strains were resistant to one or both of the tested amphenicol compounds (chloramphenicol and florfenicol). Resistance to the tested amphenicols was not observed in the corresponding transformants.

**Discussion**

Little is known about the possible relationship between plasmids harbouring bla$_{TEM-52}$. Therefore we characterized plasmids from a collection of 22 bla$_{TEM-52}$-positive isolates from animals, humans and food products originating from several different European countries as well as Canada and Korea.

Thirteen plasmids in our study carried the bla$_{TEM-52b}$ allele, while nine carried the bla$_{TEM-52c}$ allele. Both alleles were disseminated among plasmids from human and non-human isolates and they were generally associated with different plasmid incompatibility groups. Clearly the IncI1 (n=7) and IncX1A (n=9) replicons dominated among the bla$_{TEM-52}$ plasmids characterized in the study. Six of the seven IncI1 plasmids carried the bla$_{TEM-52b}$ allele. These belonged to RFLP type b and all represented ST5. One IncI1 plasmid carried the bla$_{TEM-52b}$ allele, represented RFLP type f and was found to be ST2. Curiously the six ST5 IncI1 bla$_{TEM-52b}$ plasmids originated from strains isolated in European countries, while the ST2 bla$_{TEM-52b}$ plasmid originated from Canada (human isolate). One of the ST5 IncI1 plasmids found in *E. coli* Cloeckaert TK and described in this study was originally isolated by Cloeckaert et al.7 from *S. enterica* species. Apparently epidemic bla$_{TEM-52}$ ST5 IncI1 plasmids circulated in European countries during the time between diverse strains of *E. coli* (phylotypes A, B1 and D; this study) and serovars of *S. enterica*.7 The strains were isolated from humans and poultry, indicating possible transmission of the ST5 IncI1 bla$_{TEM-52c}$/plasmid between these two reservoirs.

Nine of the 13 bla$_{TEM-52b}$ plasmids appeared identical in RFLP profiles (RFLP type a) and they shared the same IncX1A replicon. *E. coli* (phylotypes A and B1) and various *S. enterica* serovars harbouring the nine plasmids originated from poultry, poultry meat, broiler meat and beef, as well as one from a human infection. These originated from Germany, France and the Netherlands between 2001 and 2006, which demonstrates a relatively wide spread of the similar bla$_{TEM-52b}$/IncX1A plasmids among the mentioned reservoirs.

In conclusion, both the genetic content and the relative abundance of the bla$_{TEM-52}$ plasmids in 13 European countries during the period 2001–2006 confirm the wide occurrence and the impact of the aminopenicillin amp Cyl A enzymes in the *S. enterica* species.
Both the IncI1 and the IncX1A plasmids described above originated generally from diverse serovars of *S. enterica* and diverse phylogenotypes of *E. coli*. Although in some of the cases it is possible that the wild-type strains harbouring the similar plasmids were clonally related, it is clear that these very similar plasmids were capable of residing in diverse strains.

Other replicons associated with *bla*TEM-52 genes detected in this study belonged to IncI/M (*n* = 2; both carried *bla*TEM-52b), IncA/C (*n* = 1; *bla*TEM-52b), IncR (*n* = 1; *bla*TEM-52c) and IncN-like (*n* = 2; both carried *bla*TEM-52c) incompatibility families. The two IncI/M plasmids originated from different *S. enterica* serovars and they did not seem to be closely related, as their RFLP patterns were very different. The two IncN-like plasmids originated from different *E. coli* strains (phylotypes B1 and B2) isolated from Spanish poultry. Their RFLP profiles were very similar, suggesting an interspecies transmission of these similar IncN-like plasmids.

Overall, the findings described above indicate that both the *bla*TEM-52b and *bla*TEM-52c genes may be distributed on the diverse plasmid replicons, most probably due to the association with Tn3 elements. Once integrated onto the plasmid backbone, the Tn3-*bla*TEM-52 element may possibly have been transferred on that plasmid both horizontally and clonally.

Interestingly, several of the *bla*TEM-52 plasmids in this study were negative in the standard multiplex PCR for replicon typing. These replicons were sequenced and the corresponding plasmids were tested in incompatibility assays with known representatives of classical Inc families. The putative Rep protein of pE001 from *E. coli* 2161 shared 100% identity at the amino acid level with the Rep replicase of plasmid pMAS2027 and 40% identity at the amino acid level with the Rep replicase of pOLAS2. pE001 turned out to be compatible with the latter, and this could have been due to the differences between pE001 and pOLAS2 replicases. All of the remaining components of the replicon and also the transfer regions of the pE001 shared from 74% to 100% amino acid identities with pMAS2027, pOLAS2 and several other plasmids classified as IncX1-like (Figure 1). pMAS2027 was assigned by Ong et al. to the IncX1 family based solely on its sequence analysis; therefore we can only speculate that pMAS2027 could display similar incompatibility properties to the IncX1A pE001. The two plasmids share high sequence similarity with the incompatibility fragment of the classical IncX1 plasmid R485 (M11688), while the remaining IncX1 plasmids share only a partial similarity with the R485 fragment (Figure 1). The full sequence of R485 is not yet publicly available. It is highly possible that pE001 and pMAS2027 represent a separate branch of the IncX1 family termed in this study as IncX1A.

In the case of pGOC049, the results of the incompatibility assay with R46 (IncN) were difficult to interpret. Due to the similarity of pGOC049 replicase and RepB of the IncN plasmids and the lack of data on the remaining part of the pGOC049 sequence, we decided to term this plasmid as IncN-like. Another *bla*TEM-52c plasmid from *E. coli* GOC043 was found to be very similar to pGOC049. It is likely that this type of IncN-like replicon represents a separate branch of the IncN family.

The *rep* sequence of p727 shared 100% identity with *rep*Bs of IncR plasmids originating from *K. pneumoniae* (pKP245) and *E. fergusonii* (pEFER). IncR replicons apparently have a broad host range. Surprisingly, the sequences of pEFER and pKP245 do not seem to contain the functional and typical conjugative transfer elements. This could explain why the *bla*TEM-52c IncR plasmid p727 examined in the study was incapable of self-transfer in conjugation. This further indicates that IncR replicons could be mobilizable.

An important observation drawn from our study is that *bla*TEM-52 seemed to be primarily associated with a limited number of aforementioned classical replicons or replicons closely related to the classical IncX1 and IncN, but not with the IncF family. This is contrary to the *bla*TEM-52 predecessor, namely *bla*TEM-1, which is most often associated with IncFI/FIB/FIA families. The question is raised as to why *bla*TEM-52 is not observed on the same IncF scaffolds as *bla*TEM-1. If *bla*TEM-52 evolved from *bla*TEM-1, the reason could be that the *bla*TEM-52 gene did not evolve on IncF plasmids, but evolved from *bla*TEM-1 that transposed initially to IncI1, IncA/C, IncI/M, IncR or other plasmid backbones. Supporting this theory is the fact that *bla*TEM-1 as well as its evolutionary followers *bla*TEM-3, *bla*TEM-21 and *bla*TEM-24 were detected on other than IncF scaffolds. The *bla*TEM-2 gene residing on Tn3 was apparently not able to re-associate with IncF scaffolds that already harboured the Tn3 elements due to transposon immunity, thus the occurrence of this type of ESBL is limited to other mentioned replicons. Occurrence of *bla*TEM-10 and *bla*TEM-15, which are the most probable intermediates of evolution from *bla*TEM-1 to *bla*TEM-52, has been reported. However, there are no sufficient data available on replicons of plasmids carrying these. This makes the model explaining where and when the upgrade occurred from *bla*TEM-1 to the extended-spectrum *bla*TEM-52 incomplete.

Our study underlined that primarily the conjugative and relatively broad host range plasmids belonging to IncI1, IncX1A, IncA/C, IncI/M and IncN-related types are the transporters for *bla*TEM-52 genes. Also, not so commonly detected and possibly mobilizable IncR plasmids play a role in *bla*TEM-52 transmission. A conclusion drawn from our study is that IncX and IncN families of replicons might be more diverse than previously thought. In particular, the occurrence of the IncN plasmids could be generally underestimated due to the lack of suitable detection methods currently available, as the classical multiplex PCR targets only the IncX2 replicons.

Moreover, the *bla*TEM-52 plasmids were found in enteric bacteria from food-production animals, meat products and humans and in many cases they were capable of self-transfer in conjugation. Some of them conferred other than ESBL resistances to the host bacteria, namely to aminoglycosides, tetracycline and sulphonamides. This underscores the potential risk of selection for co-resistances when *bla*TEM-52 plasmids are present in enteric bacteria. The discovery of new plasmid types like IncX1A and the N-related replicons that were not detectable by means of currently available screening methods underscores the importance of further research within the area of plasmid biology, with a focus on plasmid-associated antibiotic resistance.

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Transparency declarations
None to declare.

Supplementary data
Table S1 is available as Supplementary data at JAC Online (http://jac.oxfordjournals.org/).

References