The Transferable Resistome of Produce

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ABSTRACT Produce is increasingly recognized as a reservoir of human pathogens and transferable antibiotic resistance genes. This study aimed to explore methods to characterize the transferable resistome of bacteria associated with produce. Mixed salad, arugula, and cilantro purchased from supermarkets in Germany were analyzed by means of cultivation- and DNA-based methods. Before and after a nonselective enrichment step, tetracycline (TET)-resistant Escherichia coli were isolated and plasmids conferring TET resistance were captured by exogenous plasmid isolation. TET-resistant E. coli isolates, transconjugants, and total community DNA (TC-DNA) from the microbial fraction detached from leaves or after enrichment were analyzed for the presence of resistance genes, class 1 integrons, and various plasmids by real-time PCR and PCR-Southern blot hybridization. Real-time PCR primers were developed for IncI and IncF plasmids. TET-resistant E. coli isolated from arugula and cilantro carried IncF, IncI1, IncN, IncHI1, IncU, and IncX1 plasmids. Three isolates from cilantro were positive for IncN plasmids and blaCTX-M-1. From mixed salad and cilantro, IncF, IncI1, and IncP-1/H9252 plasmids were captured exogenously. Importantly, whereas direct detection of IncI and IncF plasmids in TC-DNA failed, these plasmids became detectable in DNA extracted from enrichment cultures. This confirms that cultivation-independent DNA-based methods are not always sufficiently sensitive to detect the transferable resistome in the rare microbiome. In summary, this study showed that an impressive diversity of self-transmissible multiple resistance plasmids was detected in bacteria associated with produce that is consumed raw, and exogenous capturing into E. coli suggests that they could transfer to gut bacteria as well.

IMPORTANCE Produce is one of the most popular food commodities. Unfortunately, leafy greens can be a reservoir of transferable antibiotic resistance genes. We found that IncF and IncI plasmids were the most prevalent plasmid types in E. coli isolates from produce. This study highlights the importance of the rare microbiome associated with produce as a source of antibiotic resistance genes that might escape cultivation-independent detection, yet may be transferred to human pathogens or commensals.

KEYWORDS Escherichia coli, IncF, IncI, antibiotic resistance, horizontal gene transfer, real-time PCR

Despite its benefit to human health, consumption of produce is increasingly recognized as a source of pathogenic bacteria, antibiotic-resistant bacteria (ARB), and antibiotic resistance genes (ARGs) associated with mobile genetic elements (MGEs) (1–5). Recently, several foodborne disease outbreaks have been associated with produce contamination worldwide (5–9). The microbiome of produce is important for plant health and vigor and was shown to be highly dynamic during growth and postharvest (10), but can also contain potentially pathogenic bacteria from human and animal origins.
sources, including *Escherichia coli* strains (11). Contamination can occur preharvest (i.e., through organic fertilizers, soil, wild animals, or contaminated irrigation water) and postharvest (12, 13).

Antibiotic resistance in bacterial pathogens has increased globally due to the widespread use and misuse of antibiotics (14–17). Antibiotic resistance levels in *E. coli* are useful indicators of overall resistance levels of bacteria on foods and in animals and humans (11). Antibiotic resistance and ARGs have been documented for enteric bacteria from various types of produce, which could facilitate the dissemination of resistant bacteria to a wider community of people (1, 2, 4, 16,18, 19). If ARGs are localized on MGEs such as plasmids or conjugative transposons they can be transferred horizontally to pathogens (20). Horizontal gene transfer (HGT) takes place at sites with high cell densities of plasmid donors and recipients, nutrient availability, and selective pressure. The phytosphere, including the rhizosphere and the phyllosphere, have been reported as hot spots of HGT (21). The plasmid-mediated resistome of produce bacteria might provide the enterobacteria with ARGs in the intestine under selective conditions. Conjugative plasmids can often confer resistance not only toward multiple antibiotics but also toward heavy metal compounds or disinfectants, making coselection possible (22–25). Although plasmids belonging to the incompatibility groups IncF and IncI have a narrow host range (NHR), they are assumed to be important for the dissemination of ARGs in *E. coli* and other *Enterobacteriaceae* (26, 27). Most importantly, resistance- and virulence-associated traits of *E. coli* isolates were almost exclusively found on IncF group plasmids (28–30). However, no real-time PCR (RT-PCR) systems that allow the cultivation-independent detection and quantification of these plasmids in total community DNA (TC-DNA) are available.

In this study, culture-dependent and -independent approaches were employed to assess the transferable resistome of bacteria associated with produce (Fig. 1). We focused on tetracycline (TET) resistance because of the large amounts of tetracyclines used in animal husbandries resulting in a high load released into the agro-ecosystem via organic fertilizers (31). TET-resistant *E. coli* was isolated from produce directly after purchase and after seven days of storage by selective plating with and without prior
nonselective enrichment. In addition, transferable TET resistance plasmids were captured into *E. coli* recipient strains using the so-called exogenous plasmid isolation method (32). New real-time PCR primers were developed for the detection and quantification of IncF and IncI plasmids. TC-DNA was also extracted from the microbial fraction detached from produce or after nonselective enrichment to detect and quantify the abundance of ARGs and MGEs.

**RESULTS**

Phenotypic and genotypic characterization of TET-resistant *E. coli* isolates. To find out whether produce was a source of antibiotic-resistant *E. coli*, we determined the occurrence and resistance profiles of TET-resistant *E. coli* isolated from 24 samples of produce directly or after an overnight enrichment step. The phenotypic characterization of a total of 63 TET-resistant *E. coli* isolates from cilantro (n = 54), arugula (n = 7), and mixed salad (n = 2), of which 50 were recovered after nonselective enrichment and 13 without enrichment (20.6%) revealed an impressive diversity (Table 1).

Almost all *E. coli* isolates were resistant to antibiotics from at least one class, and two isolates were resistant to eight antibiotic classes, tetracyclines (TET and D), penicillins (AM and AMX), third generation cephalosporins (CTX and CRO), fluoroquinolones (CIP, OFX, and NA), aminoglycosides (GM and S), sulfonamides (SD), phenicols (C), and trimethoprim (TMP). Most of the TET-resistant *E. coli* also displayed resistance to ampicillin and amoxicillin (84%) and trimethoprim (73%). Resistances to ofloxacin, ciprofloxacin, sulfadiazine, and streptomycin were also common. We tested all of the isolates for the production of extended-spectrum beta-lactamases (ESBLs) with the double-disc diffusion test (DDT) and found three ESBL-producing *E. coli* which were isolated from two of the cilantro samples.

We then genotypically characterized the collection of *E. coli* isolates for the presence of various resistance genes [tet(A), strA, sul1, sul2, sul3, aadA, qacE and/or qacEΔ1 (qacE/qacEΔ1), merRTΔP, bla genes (TEM, CTX-M, and SHV), qnr genes (qnrA, qnrB, and qnrS)] and integrase genes intI1 and intI2 by RT-PCR or regular PCR of genomic DNA (Table 1). The most commonly detected ARG was the tetracycline resistance gene tet(A), which was found in 59 out of 63 isolates. A total of 10 isolates were positive for the sulfonamide resistance genes sul1, 14 for sul2, and five for sul3. The combinations of sul1 and sul2, sul2 and sul3, and sul1 and sul3 were detected in seven, three, and one isolate, respectively. All three sul genes were found in one TET-resistant *E. coli* isolate from cilantro. The qnrB and qnrS genes encoding fluoroquinolone resistance were detected alone or in combination in one and 38 isolates, respectively. The *bla*TEM genes encoding resistance to ampicillin and amoxicillin were detected in 82.5% of TET-resistant *E. coli* isolates. The *bla*CTX-M-1 gene encoding ESBL resistance was detected in only three isolates and was found in combination with *bla*TEM genes in two *E. coli* from cilantro. The *bla*SHV gene encoding ESBL resistance was not detected in any of the isolates. For the streptomycin/spectinomycin resistance genes, *aadA* (24 isolates) was most common, followed by *strA* (21 isolates) and *aadA* and *strA* (three isolates).

The class 1 integron integrase gene intI1 was detected in 50 isolates, while the class 2 integron integrase gene intI2 was not detected at all. Although qacEΔ1 encoding quaternary ammonium compound resistance is a typical component of class 1 integrons, the qacE and/or qacEΔ1 genes were detected in only 23 isolates, suggesting a large proportion of atypical class 1 integrons. Interestingly, merRTΔP encoding regulation, transport, and extracellular mercury-binding was detected in 12 isolates. These findings show that produce can be a source of multidrug-resistant *E. coli* isolates.

Characterization of plasmids in TET-resistant *E. coli* isolates. To test if the TET-resistant *E. coli* isolates recovered from produce harbor plasmids and to assign them to known plasmid groups, their genomic DNA was screened by TaqMan probe-based RT-PCR systems for IncF and IncI plasmids and by PCR-based replicon typing (PBRT) (Table 1). All isolates that were positive by RT-PCR targeting the IncF (traI gene) were also identified by replicon typing as IncF, confirming the specificity of the novel TaqMan RT-PCR system. However, PBRT also allowed assignment to the different IncF
| E. coli isolatesa | Sample sourceb | Time point (day) | Inc groupc | bla genes | Resistance and integrase genes | Antibiotic resistance profiled |
|------------------|----------------|-----------------|-------------|-----------|-------------------------------|--------------------------------|
| EK2.15          | Ci             | 0               | III         | blaTEM     | tet(A), qnrS                  | AM, AMX, TET, CIP, OFX         |
| EK2.16          | Ci             | 0               | III         | blaTEM     | tet(A), sul2, strA             | AM, AMX, TET, S, TMP           |
| EK2.17          | Ci             | 0               | II          | blaTEM     | tet(A), sul2, strA             | AM, AMX, TEX                   |
| EK2.18          | Ci             | 0               | X1h         | blaTEM     | tet(A), sul2, strA             | AM, AMX, TET, S                |
| EK2.19          | Ci             | 0               | I1i         | blaTEM     | tet(A), sul2, strA, qnrS       | AM, AMX, TET, S, TMP, SD, D    |
| EK2.20          | Ci             | 0               | I1i         | blaTEM     | tet(A), sul2, strA             | AM, AMX, TET, S, TMP, SD, D    |
| EK2.21          | Ci             | 0               | X1i         | blaTEM     | tet(A), sul2, strA             | AM, AMX, TET, S                |
| EK2.22          | Ci             | 0               | I1h         | blaTEM     | tet(A), sul2, strA, qnrS       | AM, AMX, TET, S, TMP, SD, D    |
| EK2.23          | Ci             | 0               | I1i         | blaTEM     | tet(A), sul2, strA             | AM, AMX, TET, S, TMP, SD, D    |
| EK2.24          | Ci             | 0               | I1i         | blaTEM     | tet(A), sul2, strA             | AM, AMX, TET, S, TMP, SD, D    |
| EK2.25          | Ci             | 0               | I1i         | blaTEM     | tet(A), sul2, strA             | AM, AMX, TET, S, TMP, SD, D    |
| EK2.26          | Ci             | 0               | I1i         | blaTEM     | tet(A), sul2, strA, qnrS       | AM, AMX, TET, S, TMP, SD, D    |
| EK2.27          | Ci             | 0               | I1i         | blaTEM     | tet(A), sul2, strA, qnrS       | AM, AMX, TET, S, TMP, SD, D    |
| EK2.28          | Ci             | 0               | X1h         | blaTEM     | tet(A), sul2, strA             | AM, AMX, TET, S, TMP, SD, D    |
| EK2.29          | Ci             | 0               | I1i         | blaTEM     | tet(A), sul2, strA, qnrS       | AM, AMX, TET, S, TMP, SD, D    |
| EK2.30          | Ci             | 0               | U1h         | blaTEM     | tet(A), sul2, strA             | AM, AMX, TET, S, TMP, SD, D    |
| EK2.31          | Ci             | 0               | X1h         | blaTEM     | tet(A), sul2, strA             | AM, AMX, TET, S, TMP, SD, D    |
| EK2.32          | Ci             | 0               | I1i         | blaTEM     | tet(A), sul2, strA, qnrS       | AM, AMX, TET, S, TMP, SD, D    |
| EK2.33          | Ci             | 0               | I1i         | blaTEM     | tet(A), sul2, strA             | AM, AMX, TET, S, TMP, SD, D    |
| EK2.34          | Ci             | 0               | X1i         | blaTEM     | tet(A), sul2, strA             | AM, AMX, TET, S, TMP, SD, D    |
| EK2.35          | Ci             | 0               | I1i         | blaTEM     | tet(A), sul2, strA             | AM, AMX, TET, S, TMP, SD, D    |
| EK2.36          | Ci             | 0               | X1i         | blaTEM     | tet(A), sul2, strA             | AM, AMX, TET, S, TMP, SD, D    |
| EK2.37          | Ci             | 0               | I1i         | blaTEM     | tet(A), sul2, strA, qnrS       | AM, AMX, TET, S, TMP, SD, D    |
| EK2.38          | Ci             | 0               | I1i         | blaTEM     | tet(A), sul2, strA             | AM, AMX, TET, S, TMP, SD, D    |
| EK2.39          | Ci             | 0               | I1i         | blaTEM     | tet(A), sul2, strA             | AM, AMX, TET, S, TMP, SD, D    |
| EK2.40          | Ci             | 0               | I1i         | blaTEM     | tet(A), sul2, strA, qnrS       | AM, AMX, TET, S, TMP, SD, D    |
| EK2.41          | Ci             | 0               | I1i         | blaTEM     | tet(A), sul2, strA, qnrS       | AM, AMX, TET, S, TMP, SD, D    |
| EK2.42          | Ci             | 0               | I1i         | blaTEM     | tet(A), sul2, strA             | AM, AMX, TET, S, TMP, SD, D    |
| EK2.43          | Ci             | 0               | I1i         | blaTEM     | tet(A), sul2, strA             | AM, AMX, TET, S, TMP, SD, D    |
| EK2.44          | Ci             | 0               | I1i         | blaTEM     | tet(A), sul2, strA, qnrS       | AM, AMX, TET, S, TMP, SD, D    |
| EK2.45          | Ci             | 0               | I1i         | blaTEM     | tet(A), sul2, strA             | AM, AMX, TET, S, TMP, SD, D    |
| EK2.46          | Ci             | 0               | I1i         | blaTEM     | tet(A), sul2, strA, qnrS       | AM, AMX, TET, S, TMP, SD, D    |
| EK2.47          | Ci             | 0               | I1i         | blaTEM     | tet(A), sul2, strA             | AM, AMX, TET, S, TMP, SD, D    |
| EK2.48          | Ci             | 0               | I1i         | blaTEM     | tet(A), sul2, strA, qnrS       | AM, AMX, TET, S, TMP, SD, D    |
| EK2.49          | Ci             | 0               | I1i         | blaTEM     | tet(A), sul2, strA             | AM, AMX, TET, S, TMP, SD, D    |
| EK2.50          | Ci             | 0               | I1i         | blaTEM     | tet(A), sul2, strA, qnrS       | AM, AMX, TET, S, TMP, SD, D    |

aD, direct plating; E, enrichment.
bCilanto: MS, mixed salad; A, arugula.
cDetected by RT-PCR and PBRT; f, detected by RT-PCR; g, detected by PCR; h, detected by PBRT; k, conjugal transfer into E. coli CV601; ND, not detected.
dAM, ampicillin; AMX, amoxicillin; TET, tetracycline; CIP, ciprofloxacin; OFX, ofloxacin; S, streptomycin; TMP, trimethoprim; SD, sulfadiazine; D, doxycycline; GM, gentamicin; KM, kanamycin; C, chloramphenicol; NA, nalidixic acid; CRO, ceftriaxone; CTX, cefotaxime.

**TABLE 1** Characterization of representative tetracycline-resistant *E. coli* isolates from produce

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subgroups. Furthermore, other plasmids were also identified by PBRT or RT-PCR (korB, specific for IncP-1 plasmids) or PCR (IncN). A summary of the plasmid/replicon types detected among the 63 representative TET-resistant E. coli isolates is given in Table 1. For cilantro and arugula, almost all TET-resistant E. coli isolates contained plasmids (61 out of 63), but the plasmids detected in the two isolates from mixed salad could not be assigned using RT-PCR or PBRT. In most isolates (n = 45), one plasmid type was detected, but some had two (n = 15) or three (n = 1) plasmids. Plasmids from seven different Inc groups were found in the 63 E. coli isolates, IncFII (n = 21), IncI1 (n = 17), IncX1 (n = 11), IncFIB (n = 10), IncU (n = 6), IncN (n = 4), and IncHI1 (n = 2). All Inc groups were found in E. coli isolates from cilantro, whereas only two Inc groups were found in isolates from arugula, IncI1 (n = 5) and IncFII (n = 2). Plasmids of the IncF groups (FII and FIB) were the predominant types, followed by IncI1 and IncX1 plasmids. The combination of replicon types IncFII and IncFIB was detected in two isolates, whereas the combination of replicon types IncFII and IncI1 and the combination of IncFIB and IncI1 were found in six and five isolates, respectively. In one isolate from cilantro, the combination IncFII, IncFIB and IncI1 was detected. IncI2 plasmids were not detected in any of the E. coli isolates.

**Conjugal transfer of antibiotic resistance.** Conjugation experiments were conducted in order to determine the potential transfer of antibiotic resistances to other bacteria. Conjugal transfer experiments were performed using TET-resistant E. coli isolates positive for ESBL (EK2.29, EK3.43, and EK3.44) as donors and kanamycin- and rifampin-resistant E. coli CV601 as a recipient at 37°C. We selected transconjugants on LB plates containing tetracycline and cefotaxime, which corresponded to phenotypes of the donors. The transfer of the resistance phenotypes was successful.

**Phenotypic and genotypic characterization of plasmids captured via exogenous isolation.** We further investigated the presence of transferable plasmids in produce by capturing TET resistance plasmids from nonselective enrichment cultures of fresh leaves from cilantro, mixed salad, or arugula by exogenous plasmid isolation into E. coli CV601. TET-resistant transconjugants were captured only on day 0 but not on day 7. The transfer frequencies of TET-resistant transconjugants were $1.73 \times 10^{-7}$, $1.55 \times 10^{-4}$, and $4.66 \times 10^{-9}$ per recipient in cilantro, mixed salad, and arugula, respectively. While all transconjugants obtained from cilantro (n = 27) and arugula (n = 23) were characterized, a total of only 41 transconjugants from mixed salad was analyzed due to the high number of transconjugants obtained. Based on initial phenotypic and genotypic analyses, 15 representative out of 91 TET-resistant transconjugants from produce (cilantro, n = 12; arugula, n = 1; mixed salad, n = 2) were selected for further characterization. The majority of these transconjugants acquired resistance to at least two antibiotic classes, and all were resistant to tetracycline, ampicillin, and amoxicillin. The blaTEM genes encoding ampicillin and amoxicillin resistances were detected in 86.7% of TET-resistant transconjugants (Table 2). The tetracycline resistance gene tet(A) was found in 13 out of 15 transconjugants from cilantro and arugula but not from mixed salad, while tet(Q) was detected in only one plasmid (pBMS1) isolated from the mixed salad. Four tetracycline resistance plasmids (pBC1.1, pBC1.3, pBC1.9, and pBC1.12) captured from cilantro carried the insertion sequence IS1071, class 1 integrons (intI1) and tetracycline resistance gene tet(A), but also encoded resistance to ampicillin (blaTEM) and mercury compounds (merRTΔP). Eight plasmids from cilantro transconjugants (pBC2.1, pBC2.2, pBC2.3, pBC2.4, pBC2.6, pBC2.8, pBC2.11, and pBC2.15) carried tet(A), qnrS, and blaTEM and two of the plasmids (pBC2.1 and pBC2.4) carried in addition sul1 and sul2, respectively. Two TET resistance plasmids (pBMS1 and pBMS4) captured from mixed salad carried sul1, strA, merRTΔP, blaTEM, and intI1. One plasmid (pBA1) captured from arugula carried blaTEM and tet(A) (Table 2). Thus, this approach demonstrates that transferable multidrug resistance plasmids were easily captured by E. coli CV601, a process that might also occur in the human gut.

**Identification of exogenously isolated plasmids.** The newly developed TaqMan probe-based RT-PCR assay was used to screen the TET-resistant transconjugants for the
presence of IncF and IncI plasmids and was validated by PBRT. In addition, other plasmids were also identified by RT-PCR (korB, specific for IncP-1 plasmids) and Southern blot hybridization. Plasmids of known Inc groups were detected in all transconjugants from the mixed salad and cilantro but not in the transconjugants from arugula. Representative transconjugants from cilantro and mixed salad carried either one (n = 11) or two (n = 3) replicons. In 12 transconjugants from cilantro samples, four different plasmid replicon types were detected (Table 2), IncFII (n = 3), IncFIB (n = 6), IncI1 (n = 2), and IncP-1β (n = 4). In contrast, the transconjugants isolated from mixed salad showed only one replicon type, IncFII (n = 2). One plasmid that could not be assigned by PBRT or RT-PCR was isolated from arugula leaves. The combination of replicon types IncFII and IncP-1β was detected in two transconjugants (pBC1.9 and pBC1.12), while the combination of replicon types of plasmids IncFIB and IncI1 was found in one transconjugant (pBC2.3) captured from cilantro leaves. Southern blot hybridization for sequences specific for IncP-1 plasmids revealed that four plasmids belonged to the IncP-1β subgroup. IncI2 plasmids were not detected in any TET-resistant transconjugants (Table 2). In contrast to IncFIB/FII and IncI1 plasmids, the IncP-1β plasmids captured exogenously were not detected in the 63 TET-resistant E. coli isolates.

Detection of IncF and IncI plasmids, tet(A), and intI1 in total community DNA. We also screened for plasmids (IncF, IncI1, and IncI2), tetracycline resistance gene tet(A), and integrase gene intI1 in TC-DNA extracted from bacterial communities either directly after their detachment from fresh leaves or after an enrichment step, using PCR-Southern blot hybridization and RT-PCR (Table 3). Using the RT-PCR method, IncF and IncI plasmids as well as the tet(A) gene were detected in TC-DNA extracted from enrichment cultures of leaves, but not in TC-DNA from the detached bacteria. In contrast, the intI1 gene was detected in both kinds of TC-DNA. Consistent with these results, PCR-Southern blot hybridization targeting the IncF and IncI plasmids and tet(A) revealed strong hybridization signals in TC-DNA extracted from the enrichment cultures but very weak or no signals from direct extractions.

**DISCUSSION**

The present study showed that bacteria associated with produce can carry various plasmids that might represent an important link between the environmental and the human gut microbiomes. Although initially low in abundance, TET-resistant E. coli were isolated from all purchased produce samples after nonselective enrichment. Contam-

### TABLE 2 Characterization of representative tetracycline resistant E. coli CV601 transconjugants captured from produce

| TET E. coli CV601 transconjugants<sup>a</sup> | Sample source<sup>b</sup> | Inc groups<sup>c</sup> | bla genes | Resistance, integrase genes and IS<sup>d</sup> | Antibiotic resistance profile<sup>e</sup> |
|---------------------------------------------|--------------------------|-----------------------|-----------|---------------------------------------------|----------------------------------------|
| pBC1.1                                      | CI                       | P-1β<sup>2</sup>      | **bla**<sub>TEM</sub> | intI1, tet(A), merRTΔP, qacE/qacΔ1, IS1071 | TET, AM, AMX, D                        |
| pBC1.3                                      | CI                       | P-1β<sup>2</sup>      | **bla**<sub>TEM</sub> | intI1, tet(A), merRTΔP, qacE/qacΔ1, IS1071 | TET, AM, AMX, D                        |
| pBC1.9                                      | CI                       | P-1β<sup>2</sup>, FII<sup>1</sup> | **bla**<sub>TEM</sub> | intI1, tet(A), merRTΔP, qacE/qacΔ1, IS1071 | TET, AM, AMX, D                        |
| pBC1.12                                     | CI                       | P-1β<sup>2</sup>, FII<sup>1</sup> | **bla**<sub>TEM</sub> | intI1, tet(A), merRTΔP, strA, qacE/qacΔ1, IS1071 | TET, AM, AMX, D, S                     |
| pBC2.1                                      | CI                       | FIB<sup>1</sup>       | **bla**<sub>TEM</sub> | tet(A), sul1, qnrS                          | TET, AM, AMX, D, CIP, NA, OFX, C       |
| pBC2.2                                      | CI                       | FIB<sup>1</sup>       | **bla**<sub>TEM</sub> | tet(A), qnrS                                | TET, AM, AMX, D, CIP, OFX              |
| pBC2.3                                      | CI                       | FIB<sup>1</sup>, FII<sup>1</sup> | **bla**<sub>TEM</sub> | tet(A), qnrS                                | TET, AM, AMX, D, CIP, OFX              |
| pBC2.4                                      | CI                       | FIB<sup>1</sup>       | **bla**<sub>TEM</sub> | tet(A), sul2, qnrS                          | TET, AM, AMX, D, CIP, OFX              |
| pBC2.6                                      | CI                       | FIB<sup>1</sup>       | **bla**<sub>TEM</sub> | tet(A), qnrS                                | TET, AM, AMX, D, CIP, OFX              |
| pBC2.8                                      | CI                       | FIB<sup>1</sup>       | **bla**<sub>TEM</sub> | tet(A), qnrS                                | TET, AM, AMX, D, CIP, OFX              |
| pBC2.11                                     | CI                       | FII<sup>1</sup>       | **bla**<sub>TEM</sub> | tet(A), qnrS                                | TET, AM, AMX, D, CIP, OFX              |
| pBC2.15                                     | CI                       | II<sup>1</sup>        | **bla**<sub>TEM</sub> | tet(A), qnrS                                | TET, AM, AMX, D, CIP, OFX              |
| pBMS1                                       | MS                       | FII<sup>1</sup>       | **bla**<sub>TEM</sub> | intI1, tet(Q), sul1, strA, merRTΔP           | TET, AM, AMX, D, TMP, C, S, SD        |
| pBMS4                                       | MS                       | FII<sup>1</sup>       | **bla**<sub>TEM</sub> | intI1, sul1, strA, merRTΔP                   | TET, AM, AMX, D, TMP, C, S, SD        |
| pBA1                                        | A                        | ND                    | **bla**<sub>TEM</sub> | tet(A)                                      | TET, AM, AMX, D, TMP, C, CIP          |

<sup>a</sup>Superscript r indicates resistance to the antibiotic.

<sup>b</sup>Ci, cilantro; MS, mixed salad; A, arugula.

<sup>c</sup>Detected by RT-PCR and PBRT; f, detected by RT-PCR; ND, not detected.

<sup>d</sup>IS, insertion sequence.

<sup>e</sup>TET, tetracycline; AM, ampicillin; AMX, amoxicillin; D, doxycycline; S, streptomycin; CIP, ciprofloxacin; NA, nalidixic acid; OFX, ofloxacin; C, chloramphenicol; SD, sulfadiazine.
### TABLE 3 PCR hybridization and real-time PCR of IncF, IncI1, and IncI2 plasmids and intI1 and tet(A) from TC-DNA extracted from produce before and after enrichment

| Produce          | DNA isolation | Time point (day) | IncF       | IncI1       | IncI2       | intI1     | tet(A)     |
|------------------|---------------|------------------|------------|------------|------------|-----------|------------|
| Mixed salad      | Direct extraction | 0                | RT-PCR     | Blot       | RT-PCR     | Blot      | RT-PCR     | Blot       | (-)       | (-)       | (-)       | (-)       |
|                  |               | 7                | (-)        | (-)        | (-)        | (-)       | (+)        | (+)        | (-)       | (-)       | (+)       | (-)       |
| Enrichment       |               | 0                | (+)        | (+)        | (+)        | (+)       | (+)        | (+)        | (+)       | (+)       | (+)       | (+)       |
|                  |               | 7                | (+)        | (+)        | (+)        | (+)       | (+)        | (+)        | (+)       | (+)       | (+)       | (+)       |
| Arugula          | Direct extraction | 0                | (-)        | (-)        | (-)        | (-)       | (+)        | (+)        | (-)       | (-)       | (+)       | (-)       |
|                  |               | 7                | (-)        | (-)        | (-)        | (-)       | (+)        | (+)        | (-)       | (-)       | (+)       | (-)       |
| Enrichment       |               | 0                | (+)        | (+)        | (+)        | (+)       | (+)        | (+)        | (+)       | (+)       | (+)       | (+)       |
|                  |               | 7                | (+)        | (+)        | (+)        | (+)       | (+)        | (+)        | (+)       | (+)       | (+)       | (+)       |
| Cilantro         | Direct extraction | 0                | (-)        | (-)        | (-)        | (-)       | (+)        | (+)        | (-)       | (-)       | (+)       | (-)       |
|                  |               | 7                | (+)        | (+)        | (+)        | (+)       | (+)        | (+)        | (+)       | (+)       | (+)       | (+)       |
| Enrichment       |               | 0                | (+)        | (+)        | (+)        | (+)       | (+)        | (+)        | (+)       | (+)       | (+)       | (+)       |
|                  |               | 7                | (+)        | (+)        | (+)        | (+)       | (+)        | (+)        | (+)       | (+)       | (+)       | (+)       |

Superscript numbers indicate number of positive replicates; -, not detected or no signal; (+), positive (RT-PCR); (+), medium signal; (+), strong signal.

Inoculation of produce with *E. coli* strains can occur in the field through contaminated soil (organic fertilizers), exposure to contaminated irrigation water, or during postharvest (12, 13). In this study, TET-resistant *E. coli* isolates were mostly isolated from cilantro that was purchased from supermarkets in Braunschweig and Magdeburg, Germany, followed by mixed ready-to-eat salad and arugula purchased from supermarkets in Braunschweig. This suggests that produce might be a hot spot for contamination with *E. coli* carrying multidrug resistance plasmids that occur at low abundance. A high proportion of the TET-resistant *E. coli* isolates was also resistant to penicillins (AM and AMX) and trimethoprim. Although it is difficult to compare among studies because of different methodologies used for isolation and resistance testing, our results are in line with high resistance levels to penicillins and trimethoprim previously reported for *E. coli* from irrigation water and vegetables (18), ready-to-eat salads (1), and lettuce (2). In the present study, TET resistance was commonly conferred by tet(A), partly confirming previous studies reporting tet(A) and tet(B) genes as the most common TET resistance genes in *E. coli* and *Salmonella* spp. isolated from ready-to-eat vegetables (1, 33). The rapid dissemination of tetracycline resistance among bacteria has been related not only to the occurrence of TET resistance genes on transposons and conjugative plasmids (22, 23, 34), but also to selective pressure, e.g., the use of antibiotics in animal husbandry and the spread of TET resistance genes via organic fertilizers (31).

Plasmid-mediated multidrug resistance plays an important role in the transfer of ARGs around the world (35). Our study showed that *E. coli* isolates from produce harbored various plasmids belonging to replicon types IncF, IncI1, IncX1, IncU, IncN, and IncHI1, with IncF plasmids being the most frequently detected. These plasmids might play an important role in the dissemination of antibiotic resistances. IncF plasmids were found predominantly in *E. coli* isolated from drinking water (36) and poultry farms (37). In our study, IncFII was the most frequently detected replicon type (36.5%), followed by IncFIB (15.9%), which is in line with studies on *E. coli* recovered from pigs and humans (38), wastewater (39), and animals (40). The combination of replicon types IncFII and IncFIB in two isolates is consistent with a report on *Enterobacter cloacae* from lettuce (3). However, we cannot exclude that these replicons are located on the same plasmid, as several studies have reported the combination of replicon types as a multireplicon on a single plasmid (30, 41–43), likely due to cointegration (28). In this study, TET-resistant *E. coli* isolates which carried IncF plasmids were also positive for tet(A), adaA, sul1, sul2, sul3, qacE and/or qacEDA1, qnrB, qnrS, or blaTEM genes. Previous reports found that IncF plasmids can carry genes conferring resistance to all major antibiotic classes, including aminoglycosides, β-lactams, phenicols, tetracyclines, sulfonamides, and fluoroquinolones (38, 40, 44).

The NHR IncI1 plasmid types were the second most dominant replicon type (34.9%)
and IncI1-positive isolates also carried multiple ARGs. In this study, strains carrying IncI1 plasmids were also positive for class 1 integron integrase gene intI1 and a diverse set of resistance genes, namely tet(A), sul2, strA, blaTEM, qacE and/or qacEΔ1, aadA, sul3, qnrS, and/or merR. In a recent study, IncI1 plasmids from irrigation water and lettuce carried genes sul1, tet(A), aadA, strA, and blaTEM, as well as intI1 (18). Similar phenotype and genotype profiles among E. coli strains from the current study and those recovered in previous studies from clinical samples, the environment, or other foods indicate that produce may play a potential role in the dispersal of E. coli carrying plasmid-localized ARGs. Thus, plasmids belonging to the IncF and IncI groups have the potential to be major contributors worldwide to the propagation of ARGs within enteric bacteria. One dissemination route of enteric bacteria carrying IncF and IncI plasmids might be the consumption of produce.

The newly developed TaqMan probe-based RT-PCR assays demonstrated high specificity in detecting these plasmids in E. coli isolates, and RT-PCR-positive isolates were also assigned by PBRT, which in addition enables subtyping.

This is the first study identifying NHR plasmids such as IncX1 and IncHI1 and broad-host-range (BHR) plasmid IncU in E. coli isolates recovered from cilantro leaves. Interestingly, IncX plasmids were detected in E. cloacae from lettuce (3). IncHI1 plasmids were previously reported in E. coli and Citrobacter youngae isolates from water and healthy calves, respectively (45), while the first IncU plasmids were isolated from Aeromonas salmonicida (46), and later from Aeromonas caviae from hospital effluent in the United Kingdom (47). In general, a low prevalence of ESBL-producing E. coli was found on produce, which is similar to previous studies (19, 48, 49). In the present study, ESBL-producing E. coli were isolated only from cilantro (2.8%).

To our knowledge, this is also the first report of E. coli isolates from cilantro that were positive for conjugative IncN plasmids, blaCTX-M-1, and resistance to third generation cephalosporins. The blaCTX-M-1 gene was also reported on plasmids belonging to the IncN family in E. coli isolated from farm workers, animals, humans, and the environment (50–52). Although IncN plasmids are able to replicate in a variety of Enterobactiriaeae, they are most frequently found in E. coli and Klebsiella pneumoniae, where they contribute to the dissemination of cephalosporin and carbapenem resistance (53).

The results of the present study showed that E. coli isolates harboring the blaCTX-M-1 gene also conferred resistance to at least seven classes of antibiotics tested. Moreover, E. coli harboring CTX-M genes were recently reported from lettuce and irrigation water (4, 54), raw vegetables (33, 54), and coastal waters (55, 56). Kim et al. (57) reported that ESBL-producing E. coli and Klebsiella pneumoniae carrying CTX-M were detected in ready-to-eat vegetables form a local retail market in Seoul, South Korea. A recent study has detected blaTEM genes in association with IncF and IncI plasmids from irrigation water and lettuce from 16 household farms in Estarreja, Portugal (18).

In previous reports, the occurrence of sul1 and qacEΔ1 was frequently associated with class 1 integrons (3, 58). Unexpectedly, only 27% and 36.5% of sul1- and qacE/qacEΔ1-positive isolates carried the intI1 gene, respectively, indicating that atypical class 1 integrons were more prevalent among the isolates, as previously also reported by Amos et al. (59).

In the present study, transferable TET resistance plasmids were also directly captured from the produce microbiomes on day 0 but not on day 7 after purchase, and the highest transfer frequency was observed in mixed salad, followed by cilantro and arugula. Differences in observed frequencies of transconjugants could be due to different abundances of bacteria with conjugative plasmids in the various sample types, or due to real differences in the frequencies of plasmid transfer. The latter might be affected by the metabolic activity of the produce microbiome, as plasmid transfer frequency is known to depend not only on plasmid-specific characteristics, but also on ecological factors affecting the metabolic activity of bacteria (60). Replicon types IncFil, IncFIB, IncCl1, and IncP-1β were captured from cilantro leaves, whereas only IncFII plasmids were captured from mixed salad. IncF (FII and FIB) plasmids were prevalent among TET-resistant transconjugants from both types of produce. Most of the IncF
Transferable resistome associated with produce

plasmids exogenously captured harbored \( \text{bla}_{\text{TEM}} \), \( \text{tet} \), and \( \text{qnrS} \) genes. One IncI1 plasmid was captured from cilantro, and another one was captured in combination with replicon type IncFIIB. The conjugative plasmids carried \( \text{tet} \) and \( \text{bla}_{\text{TEM}} \) genes. Finally, four IncP-1 \( \beta \) plasmids were captured from cilantro leaves and two of them in combination with replicon type IncFlII. IncP-1 plasmids have been frequently captured by exogenous plasmid isolation from various environments such as sewage sludge (61), manure (23), and water (62). However, the first isolations of IncP-1 plasmids were from clinical isolates (63, 64). The IncP-1 \( \beta \) plasmids carried genes conferring resistances to antibiotics \( \text{tet} \), \text{strA}, and \( \text{bla}_{\text{TEM}} \) and also mercury compounds (\( \text{merRT}\Delta\Phi \)) and disinfectants (\( \text{qacE/qacE}\Delta \)).

In conclusion, this study showed that produce that we eat might contain bacteria such as \( E. \text{coli} \) carrying transferable multidrug resistance plasmids. Although \( E. \text{coli} \) numbers are typically low, our nonselective enrichments showed that proliferation can easily occur. Our study reports a specific TaqMan probe-based RT-PCR assay that can be used for rapid detection of IncF and IncI plasmids in \( E. \text{coli} \) isolates and exogenously captured plasmids as well as in TC-DNA extracted from enrichment cultures of leaves. However, quantifying these plasmids in TC-DNA directly extracted from the microbial fraction detached from leaves was impossible due to their low abundance in the microbiome, but IncF and IncI plasmids were detected in DNA extracted after previous enrichment. While these assays represent an important and useful tool to be implemented for monitoring the prevalence of IncF and IncI plasmids in isolates and the environment, negative results of these and other cultivation-independent methods can lead to an underestimation of the mobile resistome present in the rare microbiome of produce and other samples. This is the first study demonstrating that multidrug resistance plasmids present in produce-associated bacteria were transferable to sensitive \( E. \text{coli} \) recipients, a process that could occur in the human gut. The NHR plasmids IncF and IncI1 and also the BHR IncP-1 \( \beta \) plasmids were captured from the produce. In particular, the captured IncF and IncI plasmids conferred resistance toward several classes of antibiotics. Thus, produce-associated bacteria should be considered an important route of disseminating transferable antibiotic resistances, which might be particularly relevant for patients under antibiotic treatment.

**MATERIALS AND METHODS**

**Sample collection.** A total of 24 samples from different locally produced or imported produce (mixed salad, arugula, and cilantro) was analyzed. The mixed salad and arugula were purchased from local supermarkets in Braunschweig, Germany, in June and September 2016, and cilantro was obtained from supermarkets in Braunschweig and Magdeburg, Germany, in May 2017. The produce was stored at refrigerator temperature and sampled on days 0 and 7 (four replicates for each time point and produce type).

**Isolation and identification of TET-resistant \( E. \text{coli} \).** For sampling, the produce was cut into pieces using a sterile scalp and mixed. For each sample, 25 g each were filled in two stomacher bags (one for direct plating and the other for enrichment) and mixed three times with 75 ml buffered peptone water (BPW; Roth, Karlsruhe, Germany), with subsequent stomacher treatment performed with the Stomacher 400 (Seward, Worthing, United Kingdom) at high speed for 1 min. The enrichment cultures of fresh leaves in BPW were incubated at 37°C with shaking (150 rpm) for 18 to 24 h. In order to isolate TET-resistant \( E. \text{coli} \), dilutions (10\(^{-1}\) and 10\(^{-2}\)) of the sample suspensions and 100 \( \mu l \) of the enrichment cultures were plated on different culture media (eosin methylene blue [EMB]; Sifin, Berlin, Germany, and Chromocult coliform agar [CCA]; Merck, Darmstadt, Germany) supplemented with tetracycline (10 mg liter\(^{-1}\)). All plates were incubated at 37°C for 18 to 24 h. The presumptive \( E. \text{coli} \) colonies were picked from each sample and streaked onto EMB, CCA, and TBX chromogenic agar (Roth, Karlsruhe, Germany) for confirmation by colony morphology and further characterization. \( E. \text{coli} \) isolates were then confirmed by biochemical tests for indole production, methyl red, and catalase activity (65). Furthermore, isolates were analyzed using PCR for the presence of the \( \text{gadA} \) gene encoding glutamate decarboxylase, specific for \( E. \text{coli} \) (66). \( E. \text{coli} \) isolates were stored in Luria broth (LB; Roth, Karlsruhe, Germany) containing 15% glycerol at −80°C.

**Exogenous plasmid isolation.** In order to capture tetracycline resistance plasmids, exogenous plasmid isolation via biparental mating was performed using \( \text{gfp}^{-} \), kanamycin (Km\(^{-}\)), and rifampin (Rif\(^{-}\))-resistant \( E. \text{coli} \) strain CV601 (67) as a recipient. The recipient strain was grown overnight in tryptic soy broth (TSB; Merck, Darmstadt, Germany) supplemented with rifampin (Rif\(^{-}\); 50 mg liter\(^{-1}\)) and kanamycin (Km\(^{-}\); 50 mg liter\(^{-1}\)). Two milliliters of the recipient strain culture was transferred into a sterile Eppendorf tube and centrifuged at 3,100 \( \times \) g for 5 min and washed twice with 1:10 TSB. Then, the pellet was resuspended in 2 ml of 1:10 TSB. The bacterial suspensions (donor) of each sample on days 0 and
7 were prepared from enrichment cultures of fresh leaves as described above. Twenty milliliters of each enrichment culture (donor) and 0.5 ml of recipient strain were mixed in a 50-ml Falcon tube. As a background control, 5 ml of the enrichment cultures and 200 μl of the recipient were processed the same way as the samples. All mixtures were centrifuged at 3,100 × g for 10 min. The pellets were resuspended in 200 μl of 1:1 TS and then spotted onto a filter for mating (Millipore filters, 0.22 μm). Filters were incubated overnight at 28°C on a plate count agar plates (PCA; Merck, Darmstadt, Germany) supplemented with cycloheximide (Cyc; 100 mg liter⁻¹). After incubation, the filters were placed in 2 ml of sterile 0.85% NaCl solution in a 50-ml Falcon tube. Each filter was washed by vortexing for 1 min. Serial 10-fold dilutions were done and appropriate dilutions were plated on PCA agar supplemented with rifampin (Rif; 50 mg liter⁻¹), kanamycin (Km; 50 mg liter⁻¹), cycloheximide (Cyc; 100 mg liter⁻¹), and tetracycline (TET; 15 mg liter⁻¹) to select for tetracycline-resistant transconjugants. Background controls of bulk soil and the recipient controls were plated on the same selective media. Numbers of recipient cells were determined by applying three replicate 20-μl drops per each serial dilution (10⁻² to 10⁻⁶) of all mating mixes on PCA with Km (50 mg liter⁻¹), Rif (50 mg liter⁻¹), and Cyc (100 mg liter⁻¹). All plates were incubated at 28°C for up to 3 days. Transconjugants were determined by green fluorescence resulting from the green fluorescence protein (GFP). The identity of putative transconjugants was confirmed by BOX-PCR (68). Transfer frequencies were calculated as total number of transconjugants divided by the total number of recipients.

**Antibiotic susceptibility testing.** Antimicrobial susceptibility testing was performed by the disk diffusion method on Müller-Hinton agar (MH; Sigma-Aldrich, St. Louis, USA), according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST). The antibiotics (μg) (Becton, Dickinson and Company, USA) used in this study were amoxicillin (25), ampicillin (10), cefotaxime (30), ceftazidime (30), ceftriaxone (30), chloramphenicol (30), ciprofloxacin (5), colistin (10), TET (30), doxycycline (30), streptomycin (10), gentamicin (10), ofloxacin (5), kanamycin (30), nalidixic acid (30), trimethoprim (5), and sulfadiazine (250). TET-resistant *E. coli* isolates were streaked onto LB agar supplemented with TET (10 mg liter⁻¹), while TET-resistant *E. coli* CV601 transconjugants were streaked on plate count agar plates (PCA; Merck, Darmstadt, Germany) supplemented with TET (15 mg liter⁻¹), Km (50 mg liter⁻¹), and Rif (50 mg liter⁻¹). *E. coli* strain CV601 was used as a negative control. The bacterial suspension was prepared from a single colony in normal saline (0.85% NaCl) to a density of 0.5 McFarland turbidity standard. Cotton swabs were used for streaking the suspension onto MH agar plates. After air drying, antibiotic discs were placed on the plates. Then all plates were incubated at 37°C for 18 to 24 h. The inhibition zone was measured. The results were interpreted according to the guidelines of EUCAST. Clinical and Laboratory Standards Institute (CLSI) recommendations were used when antibiotic breakpoints in EUCAST guidelines were absent (i.e., for doxycycline, streptomycin, tetracycline, and nalidixic acid). ESBL production was confirmed among TET-resistant *E. coli* isolates and transconjugants by double-disc diffusion test (DDT) (48). The ESBL producers were identified by phenotypic confirmatory test according to the CLSI.

**TC-DNA extraction.** The bacterial fraction detached from fresh leaves directly or after an enrichment culture of each sample as described above were pelleted by centrifugation at 3,100 × g for 15 min at 4°C. Total community DNA was extracted from the pellet using the FastDNA spin kit for soil (MP Biomedicals, Heidelberg, Germany), according to the manufacturer’s instructions. The quality of extracted DNA was determined by agarose gel electrophoresis. The extracted DNA was stored at −20°C until further analysis.

**Genomic DNA extraction.** Genomic DNA was extracted from overnight cultures of TET-resistant *E. coli* isolates, transconjugants, and the recipient strain with a Qiagen genomic DNA extraction kit (Qiagen, Hilden, Germany) using a silica-based kit (silica bead DNA extraction kit; Thermo Scientific, St. Leon-Rot, Germany). The extracted genomic DNA was stored at −20°C until further analysis.

**Primer-probe design (IncF, IncI1, and IncI2 plasmids).** As it is known that relaxase genes can be used for classification of the mobilization systems of plasmids (69), the *tra* gene region was chosen as a target region to design primers detecting IncF, IncI1, and IncI2 plasmid sequences. A total of 4,530 plasmid DNA sequences were downloaded from NCBI (NCBI, Batch Entrez) using the 4,602 plasmid accession numbers found in GenBank by Shintani et al. (70), among which 298 plasmids were identified as belonging to the MOB group. The coding sequences (CDS) of the MOB plasmids were aligned using tBLASTn against the relaxase TraI of the F plasmid (GenBank accession number AP001918), resulting in 110 protein sequences sharing >50% identity and >70% coverage. The 110 protein sequences closely related to TraI were aligned using MAFT multiple sequence alignment software version 1.3.3. The alignment produced was back translated using the EMBOSS Backtranseq tool and used to generate a set of degenerated primers and probes using Primer3. All of those steps were carried out in Geneious 8.1.9. At best, 83 of the 110 *tra* nucleic acid sequences could be targeted by one set of designed primers and probe (Table 4). Those sequences belonged mostly to plasmids isolated from *Salmonella enterica* and *Escherichia coli* and a few from *Klebsiella pneumoniae* and *Shigella* spp. The plasmids corresponded to a part of the subclade MOB⁺ defined by García-Collán-Barcia et al. (71), which comprises the phylogenetically broad IncF complex. When tested against the 4,530 plasmids, the primer/probe targeted 92 plasmids in the database, and 89 of these plasmids belonged to the MOB group (298 plasmids recovered belonged to this group) and three were annotated as “non-mob” and belonged to the IncI type. Among the 4,530 plasmid sequences, 243 carried a *rep* gene belonging to the IncF group, indicating that the primer/probe cannot detect all possible IncF plasmids.
Available IncI plasmid sequences were downloaded from NCBI. The traI genes were realigned using the software CLC Main Workbench version 8 (CLC bio, Qiagen) with standard settings for alignments, and primers were designed to match conserved regions of the traI gene (Table 4). The specificity was confirmed in silico with NCBI primer BLAST and with a set of plasmids from other incompatibility groups. Plasmids used for this test were R388, pB10, pHHV216, RSF1010, pSM1890, RP4, pHH3-414, pHH2-227, and other plasmids.

| Gene target | Primer | Primer and probe (5′-3′) | Size (bp) | Reference or source |
|-------------|--------|--------------------------|----------|---------------------|
| qacE and/or qacEΔ1 | qacEall-F | CGCATTTATTTTCTTCTCTGTT | 69 | 76 |
| | qacEall-R | CCGCCAGCAGCTGCAAAAGC | | |
| | qacEall-P | TGAATATCCATTCCGTCCTGCGT | | |
| tet(A) | tetA-qfw | TGGTCCGTGTCCTCGTA | 504 | 77 |
| | tetA_qrv | TCGGAGGATCAG | | |
| | q-tetA-P | TCGGAGGATCAG | | |
| sul1 | q-sul1 653f | CGTGTGCGCTTCTCTGAAAG | 965 | 78 |
| | q-sul1 719r | TGGCCAGCAGCTGCGT | | |
| | tp_sul1 | TGGCCAGCAGCTGCGT | | |
| sul2 | q_sul2 595f | CGGCGCGCTTCTCGAT | 865 | 78 |
| | q_sul2 654f | CGGCGCGCTTCTCGAT | | |
| | tp_sul2 614 | CGGCGCGCTTCTCGAT | | |
| sul3 | Su3-F | CAGATAAGCCATGATGCTGCTGC | 569 | 38 |
| | Su3-R | AGAATGATTCCTGACCACTGCAACCTCATT | | |
| intI | intI1-LC1 | GATCGTGGTACGCTGATGCT | 196 | 79 |
| | intI1-LC5 | GATCGTGGTACGCTGATGCT | | |
| | intI1-P | ATTCCTGGCCGTGGTTCTGGGTTTT | | |
| aadA | q-aadA-Fw | TGATCCGTCGTGTTACCTG | 635 | 80 |
| | q-aadA-Rv | CTTGATGGCTACCGCTGTTT | | |
| | q-aadA-P | TGGTATGGCTACCGCTGTTT | | |
| korB | korB-F | TCAATGGGCGGACGCTACACG | 118 | 81 |
| | korB-Fz | TCAATGGGCGGACGCTACACG | | |
| | korB-R | TCAATGGGCGGACGCTACACG | | |
| | korB-Rge | TCAATGGGCGGACGCTACACG | | |
| | korB-Rd | TCAATGGGCGGACGCTACACG | | |
| strA | q-strA-Fw | TTGATTTGCTGGTTACTGTG | 521 | 80 |
| | q-strA-Rv | CACCATGCGGACAACCATATA | | |
| | q-strA-P | CACCATGCGGACAACCATATA | | |
| intI2 | intI2-LC2 | CGGCGTACCTCCTCAGTTTCTC | 195 | 79 |
| | intI2-LC3 | CGGCGTACCTCCTCAGTTTCTC | | |
| | intI2-P | CGGCGTACCTCCTCAGTTTCTC | | |
| tet(Q) | q-tetQ-Fw | AGGTTGGCAGAGCTGTTGTCTC | 69 | 82 |
| | q-tetQ-Rv | GGGGAGCTCCGAGGAGGATT | | |
| | q-tetQ-P | TGGCATGCTACCTCCTCAGGTC | | |
| IncF (traI) | IncF_traI_Fw | TGGCATGCTACCTCCTCAGGTC | 391 | This study |
| | IncF_traI_Rev | CAGGATGATGGGCAATGCTG | | |
| | IncF_traI_TP | TGGCATGCTACCTCCTCAGGTC | | |
| | 973_P | TGGCATGCTACCTCCTCAGGTC | | |
| | IncF1 (traI) | IncF1_traI_Fw | TGGCATGCTACCTCCTCAGGTC | 118 | This study |
| | IncF1_traI_Rev | TGGCATGCTACCTCCTCAGGTC | | |
| | IncF1_traI_TP | TGGCATGCTACCTCCTCAGGTC | | |
| IncI1 (traI) | IncI1TraI_Fw | TGGCATGCTACCTCCTCAGGTC | 291 | This study |
| | IncI1TraI_Rev | TGGCATGCTACCTCCTCAGGTC | | |
| | IncI1TraI_TP | TGGCATGCTACCTCCTCAGGTC | | |
| | 973_P | TGGCATGCTACCTCCTCAGGTC | | |
| | IncI2 (traI) | IncI2TraI_Fw | TGGCATGCTACCTCCTCAGGTC | 291 | This study |
| | IncI2TraI_Rev | TGGCATGCTACCTCCTCAGGTC | | |
| | IncI2TraI_TP | TGGCATGCTACCTCCTCAGGTC | | |
| merRT1ΔP | merRT1ΔP | GGGGAGATTTAAGGAGCAGCTAGTTCA | 1000 | 83 |
| | merRT1ΔP | GGGGAGAGATTTGCGTACGAGGCGCA | | |
| | 973_P | TGGCATGCTACCTCCTCAGGTC | | |
| blaCTX-M-1 | CTX-M-F | TGGCCGGCAGCTGCGT | 908 | 84 |
| | CTX-M-R | TGGCCGGCAGCTGCGT | | |
| blaTEM | TEM-F | TGGCCGGCAGCTGCGT | 930 | 84 |
| | TEM-R | TGGCCGGCAGCTGCGT | | |
| blaSHV | SHV-F | TGGCCGGCAGCTGCGT | 796 | 84 |
| | SHV-R | TGGCCGGCAGCTGCGT | | |
| IS1071 | IS-F | GCTTGGTCCGCGGACGCTGTTTCTC | 180 | 85 |
| | IS-R | GCTTGGTCCGCGGACGCTGTTTCTC | | |
| IncN | IncN | GCTTGGTCCGCGGACGCTGTTTCTC | 180 | 85 |
| | rep-1 | GCTTGGTCCGCGGACGCTGTTTCTC | | |
| | rep-2 | GCTTGGTCCGCGGACGCTGTTTCTC | | |
| qnrA | qnrAf-RT | ATTTCCTGACCCAGATTGTT | 529 | 87 |
| | qnrAr-RT | GACATGGCAGCTGTTTCTC | | |
| qnrB | qnrBmF | GGCATGCGTCGAGGTT | 429 | 87 |
| | qnrBmR | GGCATGCGTCGAGGTT | | |
| qnrS | qnrS1nF11 | GACATGGCAGCTGTTTCTC | 393 | 87 |
| | qnrS1nR11 | GACATGGCAGCTGTTTCTC | | |

Available IncI plasmid sequences were downloaded from NCBI. The traI genes were aligned using the software CLC Main Workbench version 8 (CLC bio, Qiagen) with standard settings for alignments, and primers were designed to match conserved regions of the traI gene (Table 4). The specificity was confirmed in silico with NCBI primer BLAST and with a set of plasmids from other incompatibility groups. Plasmids used for this test were R388, pB10, pHHV216, RSF1010, pSM1890, RP4, pHH3-414, pHH2-227, and other plasmids.
pRA3, RN3, RSF1010, pT101, pTP6, R751, pQKH54, pKS308, pEST4002, pJK5, pMCBF1, pHMS149, pCAR1, pZDT, p667, pWW0, and pST527, from which none was amplified.

Detection of IncF, IncI plasmids by real-time PCR. The RT-PCR assay was performed under standard conditions. All real-time PCR (RT-PCR) reactions were set up in a 25 μl reaction volume using a Hot Start Tag DNA polymerase (M0495L, New England Biolabs, Ipswich, Massachusetts, USA) containing 5 μl of template DNA, 300 nM primer (reverse), 50 nM primer (forward), and 50 nM probe for IncF, and 300 nM primer each (forward and reverse) and TaqMan probe for IncI. All primers and probes are described in Table 4. The following PCR program was used for amplification: 10 min at 95°C, followed by 40 cycles of 95°C for 30 sec and 60°C for 1 min. The assays were carried out in triplicate with real-time PCR S1-nuclease assays (TaqMan RT-PCR) in a CFX96 real-time PCR detection system (Bio-Rad, Hercules, CA, USA). Negative controls were included in all tests, and they consisted of all the elements of the reaction except for the template DNA.

Standard plasmids were used to construct a full standard curve in duplicate in each RT-PCR run. Standard plasmids were constructed by cloning the purified PCR products amplified from the plasmids R64 for IncI1, pHNSHP45 for IncI2, and F plasmid for IncF using the corresponding primer pairs used for the RT-PCR, into TransformMax EC100 electrocompetent E. coli (Epichent), in pJET1.2 using the Thermo Scientific CloneJET PCR cloning kit (Thermo Scientific).

Detection of target genes by real-time PCR and PCR. The target genes in genomic DNA extracted from TET-resistant E. coli isolates and transconjugants were detected by RT-PCR S1-nuclease assays (TaqMan or EvaGreen RT-PCR) in a CFX96 RT-PCR detection system (Bio-Rad, Hercules, CA, USA) or by PCR for class 1 and 2 integrons; integrase genes intI1 and intI2; korB (IncP-1 plasmids); qacE and/or qacEΔ1 (tetracycline resistance genes); aadA and strA encoding streptomycin and spectinomycin resistance; tetracycline resistance genes tet(O) and tet(A); the merRT3 gene part of the mercury resistance operon; sul1, sul2, and sul3 encoding sulfonamide resistance; qnrA, qnrB, and qnrS encoding fluoroguainolone resistance; β-lactam resistance genes blaTEM, blaSHV, and blaCTX-M-1; IncN (rep); and insertion sequence (IS) IS1071 (represented by the transA gene). The DNA of the recipient strain E. coli CV601 was included as the negative control. The primers and probes targeting these genes and PCR and RT-PCR conditions are listed in Table 4.

Conjugation assay. TET-resistant E. coli isolates positive for ESBL (EK2.29, EK3.43, and EK3.44) were examined for their ability to transfer resistance. Briefly, the donors and the rifampin- and kanamycin-resistant E. coli CV601 recipient strain were grown in LB broth overnight at 37°C. 500 μl of overnight cultures of each donor and recipient strain were mixed in 1 ml LB broth and incubated at 37°C for 24 h without shaking. 100 μl of the conjugal mixture was spread on LB agar (Roth; Karlsruhe, Germany) containing Rif (50 mg liter−1), Kan (50 mg liter−1), CTX (2 mg liter−1), and TET (15 mg liter−1), and incubated at 37°C for 24 to 48 h. The transconjugants were verified by BOX-PCR and further tested for the antibiotic resistance phenotypes and genotypes as described above.

Plasmid DNA extraction and detection by Southern blot hybridization. Plasmid DNA extraction from TET-resistant transconjugants (pBC1.1, pBC1.3, pBC1.9, and pBC1.12) captured exogenously from the enrichment of cilantro leaves was performed using Qiagen Plasmid Mini Kit (Qiagen, Inc., Hilden, Germany) according to the manufacturer’s instructions. In order to detect the IncP-1 plasmids with Southern blot hybridization in these transconjugants, plasmid DNA was digested with the restriction enzyme NotI (Thermo Fisher Scientific, Waltham, MA, USA), and fragments were separated by electrophoresis on a 1% agarose-TBE gel as described previously (2). The DNA of the recipient strain E. coli CV601 was included as the negative control. The primers and probes targeting these genes and PCR and RT-PCR conditions are listed in Table 4.

Plasmid replicon typing. PBRT was used to identify the incompatibility group of plasmids in TET-resistant E. coli isolates and transconjugants, and to confirm the presence of IncF and IncI plasmids as determined via the newly developed RT-PCR method as described above. This was done by PCR amplification on genomic DNA of the strains using primer sets for 30 replicons, HI1, HI2, I1, I2, X1, X2, X3, X4, L, M, N, FIA, FIB, FIC, FII, FII, FII, FIB KN, FIB KQ, W, Y, P1, A/C, T, K, U, R, B/O, HIB-M, and FIB-M, representative of major plasmid incompatibility groups among Enterobacteriaceae (74, 28). PCR products were separated by electrophoresis on a 2.5% agarose-TBE gel and stained with ethidium bromide.

Detection of IncF and IncI plasmids, tet(A), and the class 1 integrase gene intI1 via PCR-Southern blot hybridization and RT-PCR in TC-DNA. PCR-Southern blot hybridization or RT-PCR was used to detect tetA, intI1, IncF, and IncI plasmids in TC-DNA extracted from the microbial fraction detached from leaves directly or after an enrichment step on days 0 and 7. The PCR products were separated on a 1% agarose-TBE gel electrophoresis and then transferred to a positively charged nylon membrane (GE Healthcare, UK). Southern blot hybridization was carried out with digoxigenin (DIG)-labeled probes generated from PCR amplicons, which were obtained from reference plasmids pHK5 for intI1 and tetA, as described by Dealtry et al. (75), R64 for IncI1, and pHNSHP45 for IncI2 and IncF. The primers and PCR conditions are listed in Table 4.

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