RESEARCH ARTICLE

Short-term effects of amoxicillin on bacterial communities in manured soil

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Abstract

Antibiotic-resistant bacteria, nutrients and antibiotics that enter the soil by means of manure may enhance the proportion of bacteria displaying antibiotic resistance among soil bacteria and may affect bacterial community structure and function. To investigate the effect of manure and amoxicillin added to manure on soil bacterial communities, microcosm experiments were performed with two soil types and the following treatments: (1) nontreated, (2) manure-treated, (3) treated with manure supplemented with 10 mg amoxicillin kg^{-1} soil and (4) treated with manure supplemented with 100 mg amoxicillin kg^{-1} soil, with four replicates per treatment. Manure significantly increased the total CFU count and the amoxicillin-resistant CFU count of both soil types. However, only the soil with a history of manure treatment showed a significant increase in the relative number of amoxicillin-resistant bacteria as a result of amoxicillin amendment. The majority of plasmids exogenously isolated from soil originated from soil treated with amoxicillin-supplemented manure. All 16 characterized plasmids carried the *bla*-TEM gene, and 10 of them belonged to the IncN group. The *bla*-TEM gene was detected in DNA directly extracted from soil by dot-blot hybridization of PCR amplicons and showed an increased abundance in soil samples treated with manure. Molecular fingerprint analysis of 16S rRNA gene fragments amplified from soil DNA revealed significant effects of manure and amoxicillin on the bacterial community of both soils.

Introduction

In animal husbandry, antibiotics are used worldwide not only for metaphylactic and therapeutic purposes but also for animal growth promotion (Wegener, 2003). A large proportion of the antibiotics is excreted through manure. When manure slurry is used as fertilizer, the remaining antibiotics and their metabolites enter the soil, where they might persist or reach the groundwater (Halling-Sørensen *et al.*, 1998; Jørgensen & Halling-Sørensen, 2000; Thiele-Bruhn, 2003; Karthikeyan & Meyer, 2006). Even low concentrations of antibiotics are supposed to enhance bacterial antibiotic resistance (Alonso *et al.*, 2001; Sengeløv *et al.*, 2003). Sulfonamide and tetracycline resistance genes have been detected in soil, aquatic environments and even groundwater (Chee-Sanford *et al.*, 2001; Esiobu *et al.*, 2002; Kim *et al.*, 2004). The transfer of resistant bacteria to humans may occur by means of plants if manure is used as fertilizer (Salyers, 2002). Antibiotic resistance genes can spread among soil microbial communities (Pukall *et al.*, 1996; Wiener *et al.*, 1998) by horizontal gene transfer (HGT). A major factor in HGT is the role played by mobile genetic elements (MGE), which can be plasmids, phages, integrating conjugative elements, transposons or integron gene cassettes. MGE are supposed to play a primary role in the development and dissemination of antibiotic resistance genes (Tschäpe, 1994; Witte, 1998).

Amoxicillin, which belongs to the β-lactam antibiotics, is one of the most widely used antibiotics in human and veterinary medicine (Dios *et al.*, 2006; Schweizer *et al.*, 2006; Worthy & Governale, 2006). Bacteria respond to the frequent use of amoxicillin by developing resistance, and a

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number of mechanisms of amoxicillin resistance have been described (Bush, 1997; Nagai *et al*., 2002). TEM-1 is the most commonly encountered β-lactamase in gram-negative bacteria that is able to hydrolyse penicillins, including amoxicillin (Livermore, 1995).

There are numerous studies on β-lactam antibiotic resistance genes located on MGE in clinical bacteria (Barnes *et al*., 1994; Robin *et al*., 2005; Watanabe *et al*., 2005). Although antibiotic resistance in environmental bacteria is increasingly seen as a reservoir and ecological problem (Davison, 1999), little research has been conducted on the distribution of β-lactamase-encoding genes in environmental bacteria (Henriques *et al*., 2006a, b).

In this study we aimed to investigate whether, in spite of its supposed low persistence, amoxicillin entering into soil via manure increases the levels of amoxicillin resistance and transferability and has an effect on the structural diversity of the bacterial community. Soil microcosm experiments were performed with two soil types and the following treatments: (1) nontreated soil, (2) soil treated with manure, (3) soil treated with manure supplemented with 10 mg amoxicillin kg^{-1} soil and (4) soil treated with manure supplemented with 100 mg amoxicillin kg^{-1} soil. A combination of cultivation-dependent and -independent techniques was used to explore potential short-term effects on the level and transferability of amoxicillin resistance and on the structural diversity of the soil bacteria. Exogenous isolation of MGE conferring resistance to amoxicillin was carried out to capture MGE independent from the cultivation of their original hosts (Smalla *et al*., 2000; Heuer *et al*., 2002). Molecular detection of amoxicillin resistance genes in transconjugants and in DNA directly extracted from soil focused on *bla*-TEM genes, because these genes are often localized on MGE, which can be easily transferred among bacterial cells by HGT. Furthermore, TEM-encoded β-lactamases are known to be the major resistance mechanism conferring amoxicillin resistance to bacteria. The effects of the treatments on structural diversity were investigated by denaturing gradient gel electrophoresis (DGGE) of 16S rRNA gene fragments amplified from community DNA. This is the first study concerning the effect of amoxicillin on (1) amoxicillin resistance quotients, (2) transferable amoxicillin resistance, (3) *bla*-TEM gene abundance and (4) community structure of bacteria in manured soil. A short-term study was performed because amoxicillin has a short half-life in comparison with many other antibiotics.

Materials and methods

Strains and plasmid

The rifampicin and kanamycin-resistant green fluorescent protein (*gfp*)-tagged *Escherichia coli* strain CV601 described by Heuer *et al*. (2002) was used as recipient in exogenous isolations of transferable amoxicillin resistance genes.

Plasmid pUC19 was used as positive control for *bla*-TEM detection by PCR and hybridization. The origin of plasmids pTH10 (IncP-1α), R751 (IncP-1β), RSF1010 (IncQ), RN3 (IncN) and R388 (IncW), which served in this study as positive controls for PCR-based detections and hybridization of broad-host-range (BHR) plasmids, is explained in Götz *et al*. (1996). Positive controls used for *sul* genes are described in Heuer & Smalla (2007).

Soils, manure and experimental design

Soil samples

The experiments were performed with soil material from the Ap horizon of two arable soils representative of agricultural soils in Germany, namely a silt loam (orthic luvisol, denoted soil M, from Merzenhausen) that had never been fertilized with manure, and a loamy sand from a field in which manure had been periodically applied every year (gleyic cambisol, denoted soil K, from Kaldenkirchen). Details concerning the sites and the soils are given in Heuer & Smalla (2007). The field-moist soil samples were air-dried for 3 days and sieved (2 mm) before being divided for the various treatments.

Manure

The manure for this experiment was obtained from an experimental station in Ruhlsdorf (Brandenburg, Germany) with *c*. 800 pigs. If needed, antibiotic treatment of the piglets was carried out with tetracycline. The manure was taken from the inlet pipe to the slurry tank and represents a mixed sample. The manure was stored at 10 °C until the experiment (*c*. 2 and 8 weeks before treating Merzenhausen and Kaldenkirchen soils, respectively) and was vortexed for 10 min directly before treating the soils.

The following parameters were determined for the manure: dry weight of 8.78%; pH 8.6, total N 2.72 g L^{-1}, total C 22.47 g L^{-1}, NH_4^+ 4.86 g L^{-1}, $CaCl_2$ extractable P 1.71 g L^{-1}, K^+ 3.3 g L^{-1}.

Experimental design

Amoxicillin-treated soil samples were prepared by first dissolving amoxicillin (A8523, Sigma, Steinheim, Germany) in water and then adding it to the amount of manure corresponding approximately to a general manure application for arable soils (40 mL kg^{-1} soil), and then thoroughly mixing the solution with the soils to final concentrations of 10 mg amoxicillin kg^{-1} dry soil (treatments KM10A or MM10A) or 100 mg amoxicillin kg^{-1} dry soil (treatments KM100A or MM100A) and a water content of 40% of the

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maximum water-holding capacity. Unamended (treatment KU or MU) and manure-amended (treatment KM or MM) soils served as controls to assess the effect of manure and of amoxicillin, respectively.

For each soil type and treatment, four replicate trays with 500-g aliquots were incubated aerobically at 10 °C in the dark, and the water content was held constant. Samples were collected on Days 1, 3, 8 (for Merzenhausen soil) or 9 (for Kaldenkirchen soil), and on Day 18 after manure treatment of the soils.

Cultivation-dependent analysis

One gram of soil was resuspended in 9 mL of sterile saline and vortexed, and serial dilutions were spread onto R2A medium (Merck) supplemented only with cycloheximide (100 mg L^{-1}) for total CFU counts or with 100 mg L^{-1} amoxicillin and cycloheximide (100 mg L^{-1}) for counts of amoxicillin-resistant bacteria. Plate counts were determined after incubation at 28 °C for 3 days.

Exogenous isolation of MGE carrying amoxicillin resistance genes (biparental mating)

Exogenous plasmid isolation was performed as described by Heuer *et al.* (2002). The rifampicin- and kanamycin-resistant *gfp*-tagged *E. coli* strain CV601 was used as recipient to capture MGE conferring amoxicillin resistance from soil. The recipient strain was incubated in 20 mL of Luria–Bertani broth (LB) supplemented with rifampicin (50 mg L^{-1}) and kanamycin (50 mg L^{-1}) overnight at 28 °C. Cells were harvested by centrifugation (4860 ***g***, 10 min). The pellet was washed twice with 1/10 tryptic soy broth (TSB) and finally resuspended in 50 mL of 1/10 TSB. The cell suspension was diluted to 1 : 100 and used as recipient.

One gram of soil was resuspended in 9 mL of 1/10 TSB and incubated at room temperature for 2 h. This suspension (4.5 mL) was then transferred to the new tubes, and 50 μL of recipient was added. As controls, 4.5 mL of the soil suspension without recipient added or 4.5 mL of 1/10 TSB and 50 μL of recipient were used as donor or recipient control, respectively. The tubes were centrifuged at 4860 ***g*** for 10 min, and the supernatant was discarded. The pellets were resuspended in 200 μL of 1/10 TSB and transferred to Millipore filters (0.22 μm) placed on plate count agar supplemented with cycloheximide (100 mg L^{-1}). After incubation overnight at 28 °C, the filters were resuspended in 10 mL of sterilized 0.85% NaCl. To select amoxicillin-resistant transconjugants, serial dilutions were plated on Mueller–Hinton agar (Merck) supplemented with cycloheximide (100 mg L^{-1}), rifampicin (50 mg L^{-1}), kanamycin (50 mg L^{-1}) and amoxicillin (100 mg L^{-1}). The undiluted suspensions of the filters containing the controls were applied onto the same medium. Recipient counts were performed by pipetting three times 20 μL per dilution on the medium described above but without amoxicillin. After 2 days' incubation at 28 °C, the CFU numbers of transconjugants and recipients were determined. Transconjugants were picked and streaked on Mueller–Hinton agar supplemented with rifampicin (50 mg L^{-1}), kanamycin (50 mg L^{-1}) and amoxicillin (100 mg L^{-1}). Transconjugants were confirmed by *gfp*.

Determination of transconjugant antibiotic resistance patterns

A single transconjugant colony was resuspended in sterile saline (0.85% NaCl), and 100 μL of suspension was spread with a drigalski spatula onto Mueller–Hinton agar. Paper discs (6 mm in diameter) with various antibiotics of known amounts (Becton, Dickinson and Company, France) were distributed on the inoculated plates. Resistance towards the following antibiotics was tested: amoxicillin (25 μg), sulfadiazine (SDZ 25 μg), tetracycline (TE 30 μg), streptomycin (S 10 μg), gentamicin (GM 10 μg), ampicillin (AM 25 μg), chloramphenicol (C 30 μg) and trimethoprim (TMP 5 μg). After incubation for 24 h at 28 °C, antibiotic resistance was determined by measuring the inhibition zone around the antibiotic paper discs, and comparing the inhibition zones with that of the recipient strain *E. coli* CV601. When the inhibition zone was $\leq$ 8 mm, the transconjugant strain was considered as resistant to the antibiotic.

Plasmid extraction

Plasmids from amoxicillin-resistant transconjugants were extracted using a Qiagen Plasmid Extraction Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol.

Total community DNA from soil extraction

Total community DNA (TC-DNA) was extracted from 0.5 g of soil and manure using a Fast DNA® Spin for soil kit (Bio101 by Q-Biogene) and purified with a Gene Clean® Spin kit (Bio 101) according to the manufacturer's protocol.

PCR-based detection of amoxicillin resistance genes and broad-host-range plasmids

For the PCR amplification of the *bla*-TEM gene, the PCR mix consisted of 1 × Stoffel buffer, 2.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 μmol of forward and reverse primers (Mabilat & Courvalin, 1990; Colom *et al.*, 2003), 0.05 U μL^{-1} of Taq polymerase (Applied Biosystem, Germany). One microlitre of extracted plasmid or soil DNA was used as template (about 2–5 ng μL^{-1}). The temperature profile was as follows: initial denaturation (94 °C for 7 min), 35 cycles of denaturation (94 °C for 30 s), annealing (52 °C for 30 s) and

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extension (72 °C for 1 min) and a final extension (72 °C for 7 min).

PCR of BHR plasmids was performed according to Götz *et al.* (1996) (except that 2.5 U Taq polymerase per reaction was used instead of 5 U).

Dot-blot and Southern blot analysis

Plasmids extracted from transconjugants were digested with enzymes PstI and Bst1107I, run on 1.5% agarose gels and Southern blotted. PCR products of the *bla*-TEM gene and the *rep* gene fragment of the IncN plasmid amplified from soil TC-DNA were dot-blotted. Dot and Southern blotting were performed using standard protocols (Sambrook *et al.*, 1989). Hybridization of Southern-blotted plasmid DNA or PCR products with digoxigenin-labelled probes (TEM gene, *sul* genes, IncN *rep*) was performed according to the manufacturer's instructions (Roche Diagnostic, Mannheim, Germany). All probes were generated from PCR products purified with a Gene Clean® Spin kit (Bio 101 by Q-Biogene). Probes were made for TEM, *sul1, sul2, sul3* and IncN *rep* from pUC19, R388, RSF1010, pVP440 and RN3, respectively. In the final detection step of the dot-blot hybridization of *bla*-TEM amplicons from soil TC-DNA with the digoxigenin-labelled TEM probe derived from pUC19, the chemiluminescence was measured with a luminescent image analyser, Fujifilm LAS-3000, and the intensity of the dots was recorded.

PCR and DGGE analysis of manure and soil bacterial communities

16S rRNA gene fragments (primers: F984GC, R1378) were amplified from TC-DNA as template as described by Heuer *et al.* (1997). DGGE was performed with the DCode System DGGE apparatus from Bio-Rad Inc. (Germany) as described by Heuer *et al.* (2001). Polyacrylamide gels were composed with a denaturing gradient of 26–58%, 0.17% (v/v) tetramethylethylenediamine (TEMED) and 0.047% (w/v) ammonium persulfate. PCR products were loaded on the gel either randomly or block-wise. Gels were run for 6 h, 220 V, in 0.5 × TAE at 58 °C. DNA was visualized by silver staining.

DGGE gels were transmissively scanned with high-resolution settings (Epson 1680 Pro, Seiko-Epson Corp. Suwa, Nagano, Japan). The scanned gels were then analysed with GelCompar 4.0 (Applied Maths, Ghent, Belgium) for analysis of the community fingerprints. The Pearson correlation index (*r*) for each pair of lanes within the gel was calculated as a measure of similarity between the community fingerprints. Cluster analysis was performed by applying the unweighted pair group method using average linkages (UPGMA) to the matrix of similarities.

Principal component analysis (PCA) was performed to group soil treatments according to their DGGE profiles. The band positions and their corresponding relative intensities (peak areas) retrieved from DGGE fingerprints were used to position the samples along the PC ordination axis using the software package CANOCO 4.5 (Microcomputer Power, Ithaca, NY). Before PCA, the DGGE fingerprints were processed with the software package GELCOMPAR 4.0 (Applied Maths). The background was subtracted using a rolling disc method with an intensity of 8 (relative units), and the lanes were normalized. For band searching, the sets for minimal profiling and minimal area were 5% and 0.5%, respectively. The positioning and quantification of bands present in each lane was carried out by setting tolerance and optimization at 7 points, i.e. 0.6%. Band positions that ranged within the limit determined by the tolerance setting were considered to be the same. The band positions and their corresponding intensities (surface) from each soil treatment were then exported to EXCEL. The band surface was converted to relative intensity by dividing its surface by the sum of all band surfaces in a lane.

Cloning and sequence analysis of differentiating bands

Differentiating bands from DGGE gels were excised, cloned and sequenced to identify dominant soil bacteria responding to manure and amoxicillin treatments. Bands were placed into sterilized tubes with 20 μL of MilliQ water, thoroughly ground with a pipette tip, and stored for 24 h at 4 °C to allow the DNA to diffuse out of the gel. Two microlitres of eluted DNA was used as the DNA template for 16S rRNA gene amplification, according to Heuer *et al.* (1997), and checked by DGGE to confirm that the amplicon had the same electrophoretic mobility as the excised band. Two microlitres of DNA eluted from excised bands was used for 16S rRNA gene amplification (without the GC clamp), purified and then cloned into the pGEM vector (Promega, Madison, WI) and transformed into *E. coli* JM109. Approximately 25 white colonies were picked per cloned band, resuspended in 50 μL of MilliQ water and boiled, and 1 μL was used as template for 16S rRNA gene amplification as described by Heuer *et al.* (1997). Amplicons were run on DGGE gel. Three clones that showed the same electrophoretic mobility in the DGGE gel as the excised band in the community patterns were used as template for PCR amplification with primer SP6/T7 (Promega, Mannheim, Germany). PCR products were purified and sequenced. The sequence fragments of 16S rRNA genes obtained from clones were classified according to the Naive Bayesian rRNA classifier (version 1.0) of the Ribosomal Database Project II (http://rdp.cme.msu.edu/) and compared with various sequences available in the GenBank database using BLAST-N (http://www.ncbi.nlm.nih.gov/BLAST/).

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Nucleotide sequence accession numbers

16S rRNA gene sequences cloned from DGGE gels reported in this paper have accession numbers AB281066 to AB281072 in the DDBJ/EMBL/GenBank nucleotide sequence databases.

Statistical analysis

The effects of manure and amoxicillin were analysed by ANOVA, comparing $\log(\mathrm{CFU\,g^{-1}})$ or logRQ [$\mathrm{logRQ} = \log(\mathrm{CFU\,g^{-1}})$ (RQ, resistance quotient) of amoxicillin-resistant bacteria $- \log(\mathrm{CFU\,g^{-1}})$ of total bacteria] of untreated soil with manured soil, or manured soil treated with AMX-supplemented manure, respectively. The effects of manure and amoxicillin on the abundance of TEM genes in soil were also analysed, by comparing the intensity of dots detected in TEM amplicon dot-blot hybridization for all time points of untreated soil, manured soil and amoxicillin-treated manured soil. The effect of manure and amoxicillin on soil bacterial communities was analysed by DGGE profiling of randomly loaded 16S rRNA gene products amplified from TC-DNA. The DGGE profiles were then analysed using GELCOMPAR 4.0. In this analysis, the lanes were normalized, and pairwise similarities of densitograms were determined by Pearson correlation. In this way, whole lanes were compared, instead of assigning bands and comparing their intensities or presence/absence, which can cause a severe bias for complex patterns (Rademakers & De Bruijn, 2004). The resulting similarity matrix was used to test for significant treatment effects according to Kropf *et al.* (2004). The statistical test is based on the difference between within-group similarities and between-group similarities, which should be larger for the actual samples in most cases than for random permutations if there are significant treatment effects. In all tests, significant differences were set at $P < 0.05$.

Results

Cultivation-dependent analysis

To determine whether the soil treatment with manure or with manure plus amoxicillin influenced the counts of total CFU and of CFU of amoxicillin-resistant bacteria, plate counts were performed on R2A medium without and with amoxicillin added. The CFU counts and the counts of amoxicillin-resistant bacteria in Merzenhausen soil and Kaldenkirchen soil revealed an effect of manure for both soil types (data not shown). Taking all time points together, values of $\log(\mathrm{CFU\,g^{-1}})$ of total bacteria as well as of amoxicillin-resistant bacteria were found to be significantly higher in manured soil than in untreated soil of both soil types. The manure effect became apparent only after Day 1. To test if amoxicillin had any effects on the relative abundance of resistant bacteria in soil, the difference in logRQ between manured soil and amoxicillin-treated manured soils was analysed. The average logRQ of amoxicillin-treated manured soil (10A or 100A) was usually higher than that of manured soil, but the difference was statistically significant only for Kaldenkirchen soil ($P = 0.03$, ANOVA), and not for Merzenhausen soil ($P = 0.16$, ANOVA).

The trend of increased relative numbers of resistant culturable bacteria in amoxicillin-treated manured soil lasted for 9 days in Kaldenkirchen soil, and for the whole experimental period of 18 days in Merzenhausen soil. In conclusion, manure had a clear effect on the CFU counts of total and amoxicillin-resistant bacteria in both soil types, and at least a short-term effect of amoxicillin on the proportion of amoxicillin-resistant bacteria in manured soil could be shown.

Exogenous isolation of MGE

Transferable amoxicillin resistance was captured in biparental matings with soil or manure bacteria as donor and the *gfp*-tagged *E. coli* CV601 as recipient. Transconjugants were only recovered after plating the undiluted resuspended cell mix after the mating onto amoxicillin-selective medium. A total of 29 transconjugant colonies were obtained from both soils and manure (Table 1). Most of the transconjugants from Kaldenkirchen soil were retrieved on Day 3 after the treatment. No transconjugants were retrieved from untreated Merzenhausen soil, whereas one transconjugant colony was obtained at Day 9 from untreated Kaldenkirchen soil. All other transconjugants from Kaldenkirchen soil originated from soil to which manure supplemented with amoxicillin had been added (Table 1).

To characterize the diversity of MGE conferring amoxicillin resistance to the *E. coli* recipient strain, the antibiotic resistance patterns of the 29 transconjugants obtained were determined. The number of transconjugants isolated per sample type and the antibiotic resistance pattern are summarized in Table 1. All transconjugants displayed multiple antibiotic resistances. Thirteen of the transconjugants conferred resistance to five antibiotics; five transconjugants acquired resistance to six antibiotics; and six transconjugants showed resistance to seven of the eight antibiotics tested.

At least one transconjugant for each antibiotic resistance pattern per treatment type and sampling time was further characterized. Sixteen among 29 transconjugants were chosen. Restriction patterns of plasmid DNA extracted from these 16 transconjugants with PstI and Bst1107I revealed eight distinct restriction patterns (Fig. 1a).

PCR and Southern blot hybridization of PCR amplicons and plasmid DNA

All 16 exogenously captured plasmids carried the *bla*-TEM gene, as revealed by PCR (data not shown) and by Southern

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Table 1. Transconjugants isolated from manure and both soils

	Number of transconjugants isolated from										
Antibiotic resistance pattern	MManure	MU	MM	MM10A	MM100A	KManure	KU	KM	KM10A	KM100A	Total
Amoxicillin, S, AM, TE	3					2					5
Amoxicillin, S, GM, AM, C, SDZ	1		1							2	5
Amoxicillin, S, AM, TE, SDZ	1					3	1		5	3	12
Amoxicillin, S, AM, C, TE, TMP, SDZ	2								3	1	6
Amoxicillin, AM, TE, TMP, SDZ										1	1
Total	7	0	1	0	0	5	1	0	8	7	29

MManure, manure used for Merzenhausen soil; KManure, manure used for Kaldenkirchen soil; MU/KU, Merzenhausen, Kaldenkirchen untreated soil; MM/KM, soil with manure; MM10A/KM10A, soil with manure and $10\,mg\,kg^{-1}$ soil amoxicillin added; MM100A/KM100A, soil with manure and $100\,mg\,kg^{-1}$ soil amoxicillin added. AMX, amoxicillin; SDZ, sulfadiazine; TE, tetracycline; S, streptomycin; GM, gentamicin; AM, ampicillin; C, chloramphenicol; TMP, trimethoprim.

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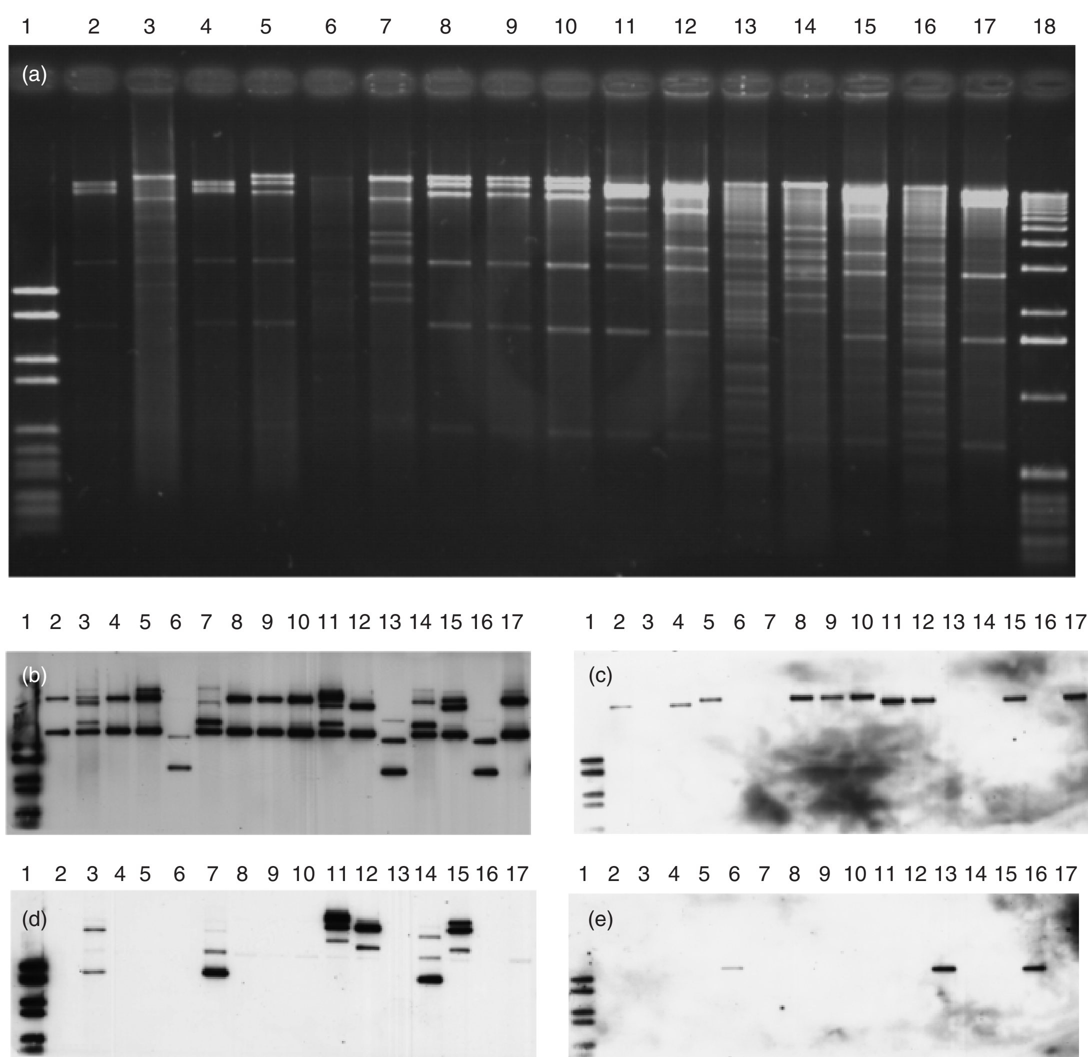

Fig. 1. Digestion patterns of plasmids extracted from 16 transconjugants, and detection of antibiotic resistance genes and BHR plasmids. (a) PstI and Bst1107I digestion patterns; Southern blot hybridization with digoxigenin-labelled probes for (b) the *bla*-TEM gene, (c) IncN, (d) *sul2* and (e) *sul3*. Lane 1: DIG-marker; lanes 2 – 6: plasmids extracted from manure used for Merzenhausen soil; lane 7: from manured Merzenhausen soil; lanes 8 – 10; from manure used for Kaldenkirchen soil; lanes 11 – 16: from Kaldenkirchen amoxicillin-treated manured soil; lane 17: from Kaldenkirchen untreated soil; lane 18 (Fig. 1a): 1-kb ladder.

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blot hybridization with the digoxigenin-labelled TEM probe generated from pUC19 (Fig. 1b). The hybridization also showed that the TEM gene was located on fragments with different sizes of the digested plasmid DNA, and that the patterns were diverse, although many of plasmids originated from the same type of sample.

Primers targetting replicon-specific sequences of IncP-1, IncN, IncW and IncQ plasmids were used to determine whether the plasmids carrying the *bla*-TEM gene were BHR plasmids. Ten of the 16 plasmids gave a product with IncN primer (data not shown), whereas no specific product (except for the positive control) was obtained with primers for IncP-1, IncQ and IncW. Southern blot hybridization of PstI/Bst1107I-digested plasmid DNA confirmed the results of the PCR screening. Ten of the 16 plasmids hybridized with the IncN *repA* probe (Fig. 1c).

According to antibiotic resistance patterns, 14 of the 16 transconjugants displayed sulfadiazine (SDZ) resistance (see Table 2). To detect plasmid carrying/conferring *sul* genes, Southern blots of digested plasmid DNA were hybridized with the *sul1*, *sul2* or *sul3* probes: the *sul1* gene was not detected on any plasmids; six plasmids gave a strong signal with the *sul2* probe (Fig. 1d); and three plasmids hybridized with the *sul3* probe (Fig. 1e). Four SDZ-resistant transconjugants gave only a very weak signal with the *sul2* probe, and one strain gave no hybridization signal with the *sul1*, *sul2* or *sul3* probe. The replicon type of the three plasmids that carry the *sul3* gene is not yet known. All information on the transconjugants is summarized in Table 2.

Detection of *bla*-TEM and IncN plasmids (*repA*) in the TC-DNA of manure and soil samples

PCR-based detection of *bla*-TEM from TC-DNA directly extracted from manures and soil samples revealed no PCR product after ethidium bromide staining the agarose gels, except for manure (data not shown). However, dot-blot hybridization of the amplicons with the *bla*-TEM probe showed that this gene was mainly detectable in manure and in the soil samples to which manure was added. Although often not all replicates per treatment gave positive hybridization signals, a significant effect of manure was detectable in Merzenhausen soil (Fig. 2; $P=0.0047$) but not in Kaldenkirchen soil ($P=0.378$). Amoxicillin did not significantly increase the abundance of TEM genes in soil ($P=0.136$ for Merzenhausen soil, and $P=0.88$ for Kaldenkirchen soil).

PCR amplification with primers targetting the IncN *repA* gene and subsequent dot-blot hybridization revealed strong hybridization signals of amplicons from manure DNA, but very weak or no signals for the various soil treatments (data not shown).

Effect of manure and amoxicillin on the soil bacterial community

To assess the effects of the various treatments on the relative abundance of dominant bacterial populations in soil, 16S rRNA gene fragments amplified from TC-DNA of Merzenhausen and Kaldenkirchen soils were loaded blockwise

Table 2. Antibiotic resistance patterns and Southern blot hybridization with probes targetting antibiotic resistance and plasmid replicon-related genes (see Figs 3 and 4) of the 16 amoxicillin-resistant transconjugants selected

			Detected genes			
No.	Transconjugant name	Antibiotic resistance pattern	*bla*-TEM	*sul2*	*sul3*	IncN
1	MManure1	S, AM,TE	+			+
2	MManure3	S, GM, AM, C, SDZ	+	+		
3	MManure4	S, AM,TE	+			+
4	MManure5	S, AM,TE, SDZ	+			+
5	MManure6	S,AM, C, TE, TMP, SDZ	+		+	
6	MM1	S, GM, AM, C, SDZ	+	+		
7	KManure1	S, AM, TE, SDZ	+	(*)		+
8	KManure2	S, AM, TE, SDZ	+	(*)		+
9	KManure3	S, AM, TE, SDZ	+	(*)		+
10	KM100A1b	AM, TE, TMP, SDZ	+	+		+
11	KM10A3a	S, AM, TE, SDZ	+	+		+
12	KM10A3b	S, AM, C, TE, TMP, SDZ	+		+	
13	KM100A3c	S, GM, AM, C, SDZ	+	+		
14	KM100A3d-1	S, AM, TE, SDZ	+	+		+
15	KM100A3d-4	S, AM, C, TE, TMP, SDZ	+		+	
16	KU9	S, AM, TE, SDZ	+	(*)		+

(*), very weak signal with *sul2* probe.
Transconjugants 1–5 from manure used for Merzenhausen soil; transconjugant 6 from manure-treated Merzenhausen soil, Day 1; transconjugants 7–9 from manure used for Kaldenkirchen soil; transconjugants 10–15 from amoxicillin-treated manured Kaldenkirchen soil, Days 1 and 3; transconjugant 16 from untreated soil, Day 9.

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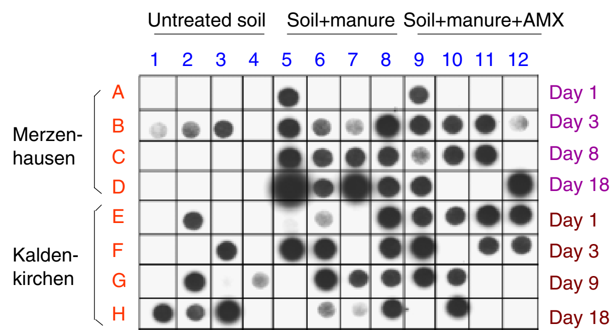
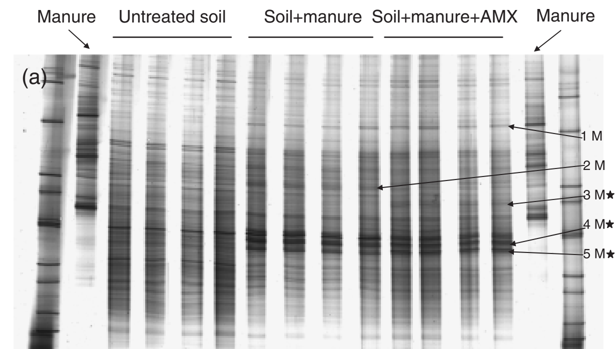
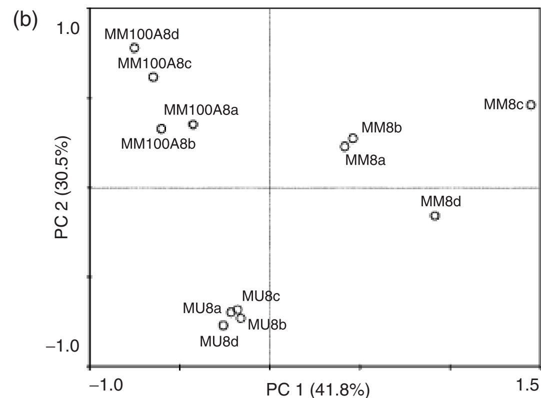


Fig. 2. Dot-blot of *bla*-TEM PCR products from soil TC-DNA hybridized with the digoxigenin-labelled *bla*-TEM probe.





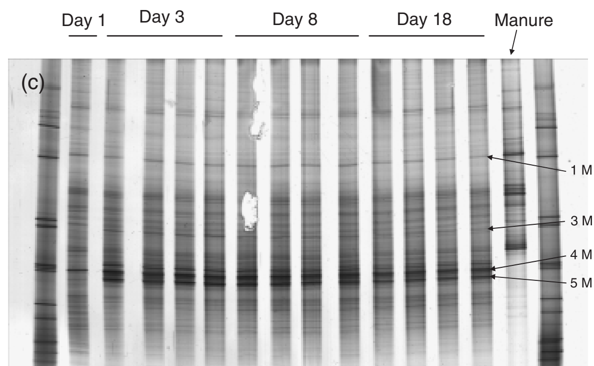


Fig. 3. DGGE analysis of bacterial 16S rRNA gene fragments amplified from TC-DNA extracted from manure and Merzenhausen soil (soil samples of four replicated microcosms per treatment, except Day 1). (a) Bacterial DGGE patterns of all treatments at Day 8, and (b) PCA based on DGGE profile. (c) Bacterial DGGE patterns of amoxicillin-treated manured soil (100 mg kg^{-1} of soil) from Days 1 to 18. The arrows indicate bands of ribotypes responding to the treatments. The bands excised for cloning are labelled with a star.

(Figs 3a and 4a) and randomly (data not shown) on DGGE gels. For both soils, some bands became stronger in the treatments with manure added. Some of these bands were also detectable in the manure patterns. In the DGGE patterns of Merzenhausen soil from Day 8, at which a significant effect of amoxicillin on CFU counts was detected (Fig. 3a), Band 1M was clearly detected in the treatments with manure, and a band with the same electrophoretic mobility was also detected in the manure patterns. Bands 4M and 5M appeared to be much stronger in the patterns of manure-treated Merzenhausen soil than those in untreated soil, but corresponding bands were not detectable in the manure DGGE fingerprints. In Kaldenkirchen soil from Day 1, at which the highest resistance quotient was observed (Fig. 4a), two strong bands that were detected in the manure patterns appeared in the soil patterns after the addition of manure (Bands 1K and 5K). Ribotypes responding to amoxicillin treatment were observed for both soils: in the patterns of Merzenhausen soil 8 days after the treatment, Band 2M became faint and Band 3M appeared only in the amoxicillin-treated manured soil in comparison with untreated soil and manured soil (Fig. 3a). In the bacterial patterns of Kaldenkirchen soil from Day 1, Band 2K seemed to disappear, and Bands 3K and 4K became very faint in the presence of amoxicillin (Fig. 4a).

To evaluate the duration of the effects of manure and amoxicillin on dominant soil bacteria, amoxicillin-treated manured Merzenhausen soil (100 mg kg^{-1} of soil) from Day 1 to Day 18 and all treatments of Kaldenkirchen soil on Day 18 were analysed (Figs 3c and 4c). The DGGE patterns of amoxicillin-treated Merzenhausen soil from Day 1 to Day 18 (Fig. 3c) showed that Band 3M, which was detected in the DGGE patterns of all manured soils at Day 8, could be observed 3 days after the treatment and was still detectable at Day 18.

The response of the bacterial communities in Merzenhausen soil to the treatment with manure was detectable until Day 18, owing to the clear presence of Bands 1M, 4M, 5M, which were very faint in the patterns of untreated soil samples. The effect of manure and amoxicillin on the relative abundance of bacterial ribotypes was also detectable in the DGGE patterns of Kaldenkirchen soil until Day 18 after the treatment (Fig. 4c).

PCA of both blockwise (see Fig. 3b) and randomly loaded gels revealed a clear clustering of the various treatments for the DGGE profiles obtained for Merzenhausen soil 8 days

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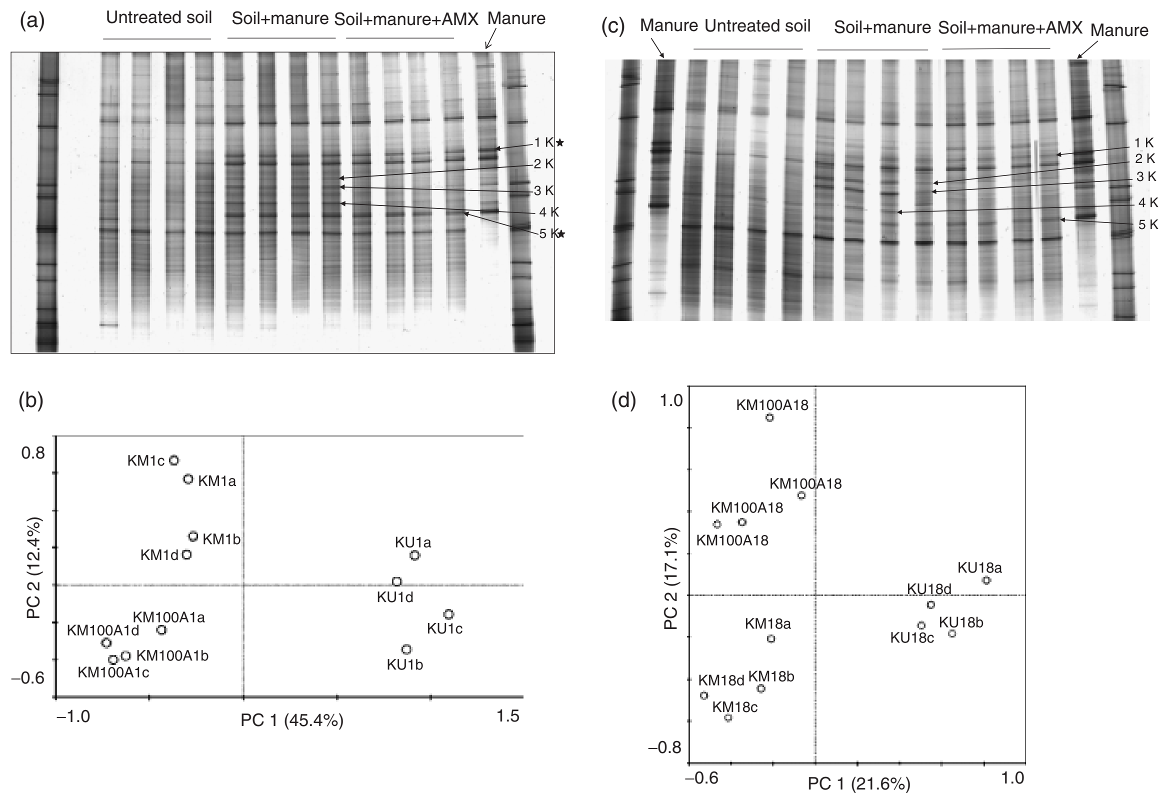

Fig. 4. DGGE analysis of bacterial 16S rRNA gene fragments amplified from TC-DNA extracted from manure and Kaldenkirchen soil (soil samples of four replicated microcosms per treatment) (a) Bacterial DGGE patterns of all treatments at Day 1, and (b) PCA based on DGGE profile. (c) Bacterial DGGE patterns of all treatments at Day 18. (d) PCA based on DGGE profile of all treatment at Day 18-replicates were randomly loaded. The arrows indicate bands of ribotypes responding to the treatments. The bands excised for cloning are labelled with a star.

after treating the soil with manure or with manure and amoxicillin. Significance testing on DGGE profiles of randomly loaded samples confirmed that both the treatment with manure and the addition of amoxicillin had a significant effect on the relative abundance of dominant ribotypes ($P = 0.0024$ and 0.0324, respectively). This effect seemed to continue until Day 18 after the treatment (Fig. 3c), because the statistical analysis performed for randomly loaded amplicons revealed that the bacterial DGGE profiles of amoxicillin-treated manured Merzenhausen soil from Day 3 to Day 18 were not significantly different. The Kaldenkirchen soil was analysed at Day 1 after the treatment (Fig. 4a), and, while all manure-treated samples were distantly clustered from the untreated samples (Fig. 4b), the profiles of manure-treated samples with amoxicillin added or not were very similar. This was confirmed by the permutation test, which was performed for the randomly loaded profiles. Whereas manure had a significant effect on the bacterial profiles of Kaldenkirchen soil at Day 1 after the treatment ($P = 0.004$), no significant effect was observed for the addition of amoxicillin ($P = 0.4592$). This situation changed when samples of the Kaldenkirchen soil were analysed at Day 18 (Fig. 4c). A clear clustering of the treatments was observed by PCA (Fig. 4d). The permutation test based on randomly loaded gels confirmed that both manure and amoxicillin had a significant effect ($P = 0.0005$ and 0.0284, respectively) on the bacterial community structure in Kaldenkirchen soil 18 days after the treatment.

Cloning and sequencing of differentiating bands from manured and amoxicillin-treated manured soils

Bands appearing in response to the treatments (manure and/or amoxicillin) were excised, cloned and sequenced (Figs 3a and 4a). Sequencing results are summarized in Table 3.

Bands 4M and 5M, which appeared clearly in the patterns of Merzenhausen soil treated with manure but seemed not to originate from manure, and Band 3M, which was detected only in amoxicillin-treated manured soil, were cloned and sequenced. Two clones co-migrated with Band 4M in the DGGE patterns of Merzenhausen soil. The 16S rRNA gene sequence determined for one of the clones had 100% identity with an *Arthrobacter* sp., and the other had

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Table 3. Clones and sequences of differentiating bands from manure and amoxicillin-treated manured soils excised from the DGGE gels (shown in Fig. 3a: Bands 3M, 4M, 5M and Fig. 4a: Bands 1K, 5K)

Band	Clone	Accession No.	Closest organisms and/or environmental 16S rRNA gene (% identity)	References
3M	24-3i	AB281070	Uncultured bacterium clone DBW1-51 (96%) Access. No. AY456903	Zhou *et al*. (2004)
4M	12e	AB281066	*Arthrobacter* sp. (100%)	
	12h	AB281067	*Pseudomonas* sp. (100%)	
5M	24-2b	AB281068	*P. marginalis* (100%)	
	24-2e	AB281069	*Pseudomonas lini* (100%)	
1K	45d	AB281071	Uncultured bacterium clone AF371835 (100%)	Leser *et al*. (2002)
5K	89a	AB281072	*Lactobacillus reuterii* (100%)	

100% identity with *Pseudomonas* sp. Sequences of cloned 16S rRNA gene fragments amplified from Band 5M had 100% identity with the 16S rRNA gene sequences of *Pseudomonas marginalis* and *Pseudomonas lini*. Only one clone comigrating with Band 3M was obtained. The 16S rRNA gene sequence had 96% identity with uncultured bacterium clone DBW1-51 (accession no. AY456903). Bands 1K and 5K in Kaldenkirchen soil patterns, for which co-migrating bands were detected in the manure patterns, were also chosen for cloning and sequencing. Band 1K in Kaldenkirchen soil had 100% identity with uncultured bacterium clone AF371835 (Leser *et al*., 2002) from the pig gastrointestinal tract. Band 5K had 100% sequence identity with the 16S rRNA gene sequence from *Lactobacillus reuterii*.

Discussion

Along with tetracyclines and sulfonamides, amoxicillin is one of the most frequently used antibiotics in animal husbandry. However, amoxicillin is supposed to be less stable and persistent in the environment than tetracycline and sulfonamide compounds. This study aimed to explore whether treatments of soil with manure or with manure with amoxicillin added – despite the low persistence of amoxicillin – result in an increased abundance of amoxicillin resistance and transferability as well as in changes of the structural composition of the bacterial community. Recently, Heuer & Smalla (2007) showed that SDZ introduced by means of manure into soil increased SDZ resistance quotients as well as the abundance of resistance genes and their transferability. The effects observed differed depending on the soil type. In both studies the antibiotic was added to the manure before soil amendments. Thus the effects of the antibiotics on the intestinal flora and a putative activation of HGT processes as well as the appearance of metabolites could not be considered.

In contrast to the study by Heuer & Smalla (2007), the potential effects of amoxicillin and manure on the soil bacterial community were monitored for only 18 days, because amoxicillin can be detected in soil only for a fairly short time (M. Lamshöft and M. Spiteller, pers. commun.). A high proportion of bacteria with the amoxicillin resistance phenotype was observed, even for untreated soils. Manure treatment enhanced not only the total bacterial CFU counts but also the CFU counts of amoxicillin-resistant bacteria. Whereas SDZ was reported to increase SDZ resistance quotients significantly even after 2 months, a significant effect of amoxicillin on amoxicillin resistance quotients was only seen for Kaldenkirchen soil. Thus, the effects of amoxicillin on the culturable fraction of soil bacteria were clearly less pronounced than the effects seen with SDZ. Considering the high proportion of amoxicillin-resistant bacteria in soil, the transferability of amoxicillin resistance was surprisingly low. The sole use of the *gfp*-tagged *E. coli* CV601 as recipient for mobile genetic elements conferring amoxicillin resistance surely limits the insights into the mobile gene pool, and merely allowed us to characterize MGE that stably replicate in *E. coli*. In fact, much higher numbers of *E. coli* CV601 transconjugants were originally picked from the selective plates for transconjugants, but a large proportion did not grow when transferred to a fresh selective medium. Thus, only 29 transconjugants were finally available for further phenotypic and genotypic characterization. Interestingly, no transconjugants were captured into *E. coli* CV601 from untreated Merzenhausen soil and only one from Kaldenkirchen soil. One of the reasons for this might be that the Kaldenkirchen soil had a history of manure treatments, whereas soil from Merzenhausen had never been treated with manure. Most transconjugants from soils were isolated 3 days after treatment from Kaldenkirchen soil amended with manure and amoxicillin. The advantage of exogenously isolated plasmids is that the acquired traits can easily be recognized, for example by comparing the antibiotic resistance patterns of the transconjugant with those of the recipient strain (Smalla & Sobecky, 2002). All transconjugants conferred multiple resistances to the *E. coli* host, with amoxicillin, streptomycin, tetracycline and gentamicin being the most frequently encoded antibiotic resistances localized on the exogenously captured plasmids. The 16 transconjugants with different antibiotic resistance patterns or with the same pattern but originating from different samples revealed a high diversity of restriction pattern types

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(8), and 10 plasmids representing four restriction fragment length polymorphism types belonged to the incompatibility group IncN. Although their host range is supposed to be less broad than that of the plasmids belonging to the incompatibility group IncP-1, IncN plasmids are considered as broad host range (Pukall *et al.*, 1996). The exogenous isolation of two plasmids conferring multiple antibiotic resistances and mercury resistance from piggery manure samples that were assigned to the IncN group has already been reported by Smalla *et al.* (2000).

The assumption that TEM genes play a primary role for amoxicillin resistance in gram-negative bacteria could also be confirmed in this study because all transconjugants, which were isolated based on the acquired amoxicillin resistance, hybridized with the TEM-gene probe generated from pUC19. Fourteen of the transconjugants conferred SDZ resistance, which was encoded in nine transconjugants by either the *sul2* gene (6) or the *sul3* gene (3). This is the first report on exogenously isolated MGE carrying the *sul3* gene, which was only recently discovered by Perreten & Boerlin (2003). Four plasmids conferring SDZ resistance gave only very weak hybridization signals with *sul2*, indicating that the genes conferring SDZ resistance in these plasmids seemed to have only a low degree of sequence identity. One plasmid conferring SDZ resistance did not hybridize with any of the *sul* probes.

Another cultivation-independent approach used in this study was the analysis of TC-DNA, which was directly extracted from soil or manure samples. PCR and hybridization revealed that the abundance of *bla*-TEM genes was strongly enhanced as a result of manure treatments, but no effect was detected for amoxicillin. Interestingly, although the majority of the plasmids exogenously isolated into *E. coli* CV601 belonged to the IncN group, PCR-based detection in soil TC-DNA showed that the abundance of the IncN plasmids was below or around the detection limit. In contrast, strong hybridization signals were observed for *bla*-TEM and IncN *repA* from manure TC-DNA. These findings indicate that manure treatment of soils increased the abundance of indigenous soil bacteria carrying *bla*-TEM genes.

DGGE analysis of PCR-amplified 16S rRNA gene fragments was used to assess potential treatment effects on the dominant bacterial populations. Loading the amplicons of the various treatments with four replicates next to each other enabled us to identify bands that only appeared in the manure treatments or in the amoxicillin-treated manured soils. Not all these bands could be detected in the manure sample. Thus, the changes detected in the composition of the dominant ribotypes seem to result not only from the introduction of manure bacteria but also from an increased abundance of some soil-derived populations, for example in response to the manure-derived nutrients. For example, Bands 4M and 5M were detected in the DGGE patterns of manure-treated Merzenhausen soil but not in manure. Sequencing identified the ribotypes behind these bands as *Pseudomonas* and *Arthrobacter*, and thus supported the assumption that the relative abundance of soil bacteria was increased as a result of manure treatment. The ribotype behind Band 3M showed an increased abundance in response to amoxicillin treatment and displayed the highest identity with the uncultured bacterium (AY456903) retrieved from sandy subsurface soil in a study by Zhou *et al.* (2004). Sequencing of Bands 1K and 5K, which were detected not only in the DGGE patterns of manure-treated Kaldenkirchen soil but also in the corresponding manure patterns and which were still detected after 18 days, revealed that the 16S rRNA gene sequence behind Band 1K had identity with an uncultured bacterium (AF371835; Leser *et al.*, 2002) from the pig gastrointestinal tract, and, according to the classifier program in the RDP database, this sequence is grouped into the genus *Clostridium* with high confidence (99%). Band 5K had 100% identity with the *Lactobacillus reuterii* sequence. Thus these ribotypes probably originated from manure and seem to colonize the soil.

To avoid an effect of the loading position on the DGGE gel, randomly loaded amplicons were used in addition to the blockwise-loaded gels to test for the significance of the effects of manure and manure–amoxicillin treatments on the soil bacterial communities. Although significance testing was carried out based on Pearson correlation indices, the results confirmed very well the data of PCA obtained with the CANOCO program based on band positions and their peak areas. PCA for both randomly and blockwise-loaded DGGE profiles revealed very similar results. The clear and rather distant clusters obtained with PCA for Merzenhausen soil at Day 8 indicated that both the addition of manure and the addition of amoxicillin had a pronounced effect on the composition of dominant bacterial ribotypes. PCA also showed that, 1 day after the treatment of Kaldenkirchen soil, no effect of amoxicillin was visible, whereas a pronounced effect of manure was detected because the ordinates of manure and manure plus amoxicillin treated samples formed no distinct clusters, and, in addition, significance testing revealed no significant effect. At Day 18 the treatments of Kaldenkirchen soil formed distinct clusters, and both treatments were shown to have a significant effect. Interestingly, the percentage of variation explained by PC1 and PC2 decreased from 57.8% at Day 1 to 38.7% at Day 18 after the treatment, which indicates what is known as soil resilience.

Although the effects of manure plus amoxicillin on the soil bacterial community were transient and less pronounced than the effects reported for SDZ introduced by means of manure into soil, effects were detectable with the various approaches used in this study, despite the predicted low persistence of amoxicillin. The diversity of exogenously

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isolated plasmids conferring resistance to a range of antibiotics was impressive. These plasmids could easily be captured by soil bacteria from the horizontal gene pool if local adaptation to antibiotics is required. Manure and amoxicillin treatments seemed to increase the MGE transferability. In conclusion, this study indicates that transferable amoxicillin resistance was introduced into soil by means of manure bacteria, and that amoxicillin transferability seemed to be enhanced by amoxicillin treatment.

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References

Alonso A, Sánchez P & Martínez JL (2001) Environmental selection of antibiotic resistance genes. *Environ Microbiol* **3**: 1–9.

Barnes AC, Hastings TS & Amyes SGB (1994) Amoxycillin resistance in Scottish isolates of *Aeromonas salmonicida*. *J Fish Diseases* **17**: 357–363.

Bush K (1997) The evolution of β-lactamases. *Antibiotic Resistance, Origins, Evolution, Selection and Spread. CIBA Foundation Symposium 20* (Chadwick DJ & Goode J, eds), pp. 152–163. John Wiley & Sons, Chichester.

Chee-Sanford JC, Aminov RI, Krapac IJ, Garrigues-Jeanjean N & Mackie RI (2001) Occurrence and diversity of tetracycline resistance genes in lagoons and groundwater underlying two swine production facilities. *Appl Environ Microbiol* **67**: 1494–1502.

Colom K, Pérez J, Alonso R, Fernández-Aranguiz A, Lariño E & Cisterna R (2003) Simple and reliable multiplex PCR assay for detection of bla_{tem}, bla_{shv}, bla_{oxa-1} genes in *Enterobacteriaceae*. *FEMS Microbiol Lett* **223**: 147–151.

Davison J (1999) Genetic exchange between bacteria in the environment. *Plasmid* **42**: 73–91.

Dios PD, Carmona IT, Posse JL, Henríquez JM, Feijoo JF & Fernández MA (2006) Comparative efficacies of amoxicillin, clindamycin, and moxifloxacin in prevention of bacteremia following dental extractions. *Antimicrob Agents Chemother* **50**: 2996–3002.

Esiobu N, Armenta L & Ike J (2002) Antibiotic resistance in soil and water environments. *Environ Health Res* **12**: 133–144.

Götz A, Pukall R, Tietze E, Prager R, Tschäpe H, van Elsas JD & Smalla K (1996) Detection and characterization of broad-host-range plasmids in environmental bacteria by PCR. *Appl Environ Microbiol* **62**: 2621–2628.

Halling-Sørensen B, Nielsen SN, Lanzky PF, Ingerslev F, Lutzhøft HCH & Jørgensen SE (1998) Occurrence, fate, and effects of pharmaceutical substances in the environment – a review. *Chemosphere* **36**: 357–393.

Henriques IS, Fonseca F, Alves A, Saavedra MJ & Correia A (2006a) Occurrence and diversity of integrons and β-lactamase genes among ampicillin-resistant isolates from estuarine waters. *Res Microbiol* **157**: 938–947.

Henriques IS, Moura A, Alves A, Saavedra MJ & Correia A. (2006b) Analysing diversity among β-lactamase encoding genes in aquatic environments. *FEMS Microbiol Ecol* **56**: 418–429.

Heuer H & Smalla K (2007) Manure and sulfadiazine synergistically increased bacterial antibiotic resistance in soil over at least two months. *Environ Microbiol* **9**: 657–666.

Heuer H, Krsek M, Baker P, Smalla K & Wellington EMH (1997) Analysis of actinomycete communities by specific amplification of genes encoding 16S rRNA and gel-electrophoretic separation in denaturing gradients. *Appl Environ Microbiol* **63**: 3233–3241.

Heuer H, Wieland G, Schönfeld J, Schönwälder A, Gomes NCM & Smalla K (2001) Bacterial community profiling using DGGE or TGGE analysis. *Environmental Molecular Microbiology: Protocols and Applications* (Rochelle PA, ed.), pp. 178–190. Horizon Scientific Press, Wymondham, UK.

Heuer H, Krögerrecklenfort E, Egan S *et al*. (2002) Gentamicin resistance genes in environmental bacteria: prevalence and transfer. *FEMS Microbiol Ecol* **42**: 289–302.

Jørgensen SE & Halling-Sørensen B (2000) Drugs in the environment. *Chemosphere* **40**: 691–699.

Karthikeyan KG & Meyer MT (2006) Occurrence of antibiotics in wastewater treatment facilities in Wascosin, USA. *Sci Total Environ* **361**: 196–207.

Kim SR, Nonaka L & Suzuki S (2004) Occurrence of tetracycline resistance genes *tet*(M) and *tet*(S) in bacteria from marine aquaculture sites. *FEMS Microbiol Lett* **237**: 147–156.

Kropf S, Heuer H, Grüning M & Smalla K (2004) Significance test for comparing complex microbial community fingerprints using pairwise similarity measures. *J Microbiol Methods* **57**: 187–195.

Leser TD, Amenuvor JZ, Jensen TK, Lindecrona RH, Boye M & Møller K (2002) Culture-independent analysis of gut bacteria: the pig gastrointestinal tract microbiota revisited. *Appl Environ Microbiol* **68**: 673–690.

Livermore DM (1995) β-lactamases in laboratory and clinical resistance. *Clin Microbiol Rev* **8**: 557–584.

Mabilat C & Courvalin P (1990) Development of oligotyping for characterization and molecular epidemiology of TEM β-lactamases in members of the family *Enterobacteriaceae*. *Antimicrob Agents Chemother* **34**: 2210–2216.

Nagai K, Davies TA, Jacobs MR & Appelbaum PC (2002) Effects of amino acid alterations in penicillin-binding proteins (pbps) 1a, 2b, and 2x on PBP affinities of penicillin, ampicillin,

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amoxicillin, Cefditoren, Cefuroxime, Cefprozil, and Cefaclor in 18 clinical isolates of penicillin-susceptible, -intermediate, and -resistant *Pneumococci*. *Antimicrob Agents Chemother* **46**: 1273–1280.

Perreten V & Boerlin P (2003) A new sulfonamide resistance gene (*sul3*) in *Escherichia coli* is widespread in the pig population of Switzerland. *Antimicrob Agents Chemother* **47**: 1169–1172.

Pukall R, Tschäpe H & Smalla K (1996) Monitoring the spread of broad host and narrow host range plasmids in soil microcosms. *FEMS Microbiol Ecol* **20**: 53–66.

Rademaker JLW & De Bruijn FJ (2004) Computer-assisted analysis of molecular fingerprint profiles and database construction. *Molecular Microbial Ecology Manual* (Kowalchuk GA, de Bruijn FJ, Head IM, Akkermans ADL & van Elsas JD, eds), pp. 1397–1446. Kluwer Academic Publishers, Dordrecht, The Netherlands.

Robin F, Delmas J, Chanal C, Sirot D, Sirot J & Bonnet R (2005) TEM-109 (CMT-5), a natural complex mutant of TEM-1β-Lactamase combining the amino acid substitutions of TEM-6 and TEM-33 (IRT-5). *Antimicrob Agents Chemother* **49**: 4443–4447.

Salyers AA (2002) An overview of the genetic basis of antibiotic resistance in bacteria and its implications for agriculture. *Animal Biotechnol* **13**: 1–5.

Sambrook J, Fritsch EF & Maniatis T (1989) *Molecular Cloning: A Laboratory Manual*, 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Schweizer G, Ehrensperger F, Torgerson PR & Braun U (2006) Clinical findings and treatment of 94 cattle presumptively diagnosed with listeriosis. *Vet Rec* **158**: 588–592.

Sengeløv G, Agersø Y, Halling-Sørensen B, Baloda SB, Andersen JS & Jensen LB (2003) Bacterial antibiotic resistance levels in Danish farmland as a result of treatment with pig manure slurry. *Environ Int* **28**: 587–595.

Smalla K & Sobecky PA (2002) The prevalence and diversity of mobile genetic elements in bacterial communities of different environmental habitats: insights gained from different methodological approaches. *FEMS Microbiol Ecol* **42**: 165–175.

Smalla K, Heuer H, Götz A, Niemeyer D, Krögerrecklenfort E & Tietze E (2000) Exogenous isolation of antibiotic resistance plasmids from piggery manure slurries reveals a high prevalence and diversity of incQ-like plasmids. *Appl Environ Microbiol* **66**: 4854–4862.

Thiele-Bruhn S (2003) Pharmaceutical antibiotic compounds in soil – a review. *J Plant Nutr Soil Sci* **166**: 145–167.

Tschäpe H (1994) The spread of plasmids as a function of bacterial adaptability. *FEMS Microbiol Ecol* **15**: 23–32.

Watanabe K, Tanaka A, Imase K, Tokunaga K, Sugano H, Kai A, Ishida H, Itoh T & Takahashi S (2005) Amoxicillin resistance in *Helicobacter pylori*: studies from Tokyo, Japan from 1985 to 2003. *Helicobacter* **10**: 4–11.

Wegener HC (2003) Antibiotics in animal feed and their role in resistance development. *Curr Opin Microbiol* **6**: 439–445.

Wiener P, Egan S & Wellington EMH (1998) Evidence for transfer of antibiotic-resistance genes in soil populations of streptomycetes. *Mol Ecol* **7**: 1205–1216.

Witte W (1998) Medical consequences of antibiotic use in agriculture. *Science* **279**: 996–997.

Worthy KC & Governale LA (2006) Trends in outpatient prescription usage in the U.S., 2002–2005. 12th Annual FDA Science Forum, April 18–20, 2006 (http://www.accessdata.fda.gov/).

Zhou J, Xia B, Huang H, Palumbo AV & Tiedje JM (2004) Microbial diversity and heterogeneity in sandy subsurface soils. *Appl Environ Microbiol* **70**: 1723–1734.

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