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Amplification and attenuation of tetracycline resistance in soil bacteria: aquifer column experiments

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Abstract

A growing inefficacy of antimicrobial agents to treat infectious diseases has stimulated research on the development of antibiotic resistance in bacteria in the environment. Sustained exposure of soil microorganisms to tetracycline (TC) in flow-through columns (50 mg/L influent) significantly decreased the effluent concentration of total heterotrophs and selected for TC-resistant (Tet^r) soil bacteria. This suggests that TC released to the environment from animal farms may contribute to the development and amplification of TC resistance, with soil bacteria serving as reservoirs for antibiotic resistance continuance. *Burkholderia cepacia*, with genetic determinants for efflux pumps that facilitate TC excretion, was the only bacterium that grew on TC-amended R2A plates. Following 300 days of exposure, TC was removed from the influent to study the recovery pattern of the microbial community. The percentage of Tet^r hererotrophs decreased from 25% to close to the control level of 1% within 1 month of discontinuing TC exposure. This was due both to a significant rebound in the total heterotrophic population and to a significant decrease in the concentration of Tet^r bacteria. Thus, discontinuing TC exposure or curtailing its use should enhance natural attenuation mechanisms that mitigate the spread of resistance vectors.

Keywords: Antibiotic resistance; *Burkholderia cepacia*; Efflux pump determinants; Tetracycline

1. Introduction

Antibiotic resistance is a public health concern of great urgency due to a growing inefficacy of antimicrobial agents to treat infectious diseases. This is mainly due to the propagation of antibiotic resistance genes among bacteria, which is exacerbated by the potential overuse of antimicrobials in humans and the intensive use of antibiotics in animal agriculture (Mellon et al., 2001). Approximately 24.6 million pounds of antibiotics are used annually in animal agriculture (70% of which is used for non-therapeutic purposes such as growth promotion and disease prevention), compared to only 3 million pounds per year used in human medicine (Mellon et al., 2001). A significant fraction of the antibiotics fed to animals (25–75%) are excreted unaltered in feces, and persist in soil after land application (Donoho, 1984; Elmund et al., 1971; Galvalchin and Katz, 1994). Recent studies have found that antibiotic resistance genes develop in bacteria in the environment as a direct result of animal agriculture (e.g., swine production facilities and fish farm), and that soil and groundwater in the vicinity of such facilities may be potential sources of antibiotic resistance in the food chain (Chee-Sanford et al., 2001). Therefore, antibiotic resistance could be considered as an environmental

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pollution problem, with resistance gene vectors as the target pollutants.

Since the isolation of the first tetracycline-resistant (Tet^r) bacterium (*Shigella dysenteriae*, isolated in 1953), tetracycline (TC) resistance has been detected in a wide variety of bacteria (Roberts, 1996). Three main mechanisms of microbial resistance to TC have been identified. First, microbial production of ribosomal protection protein (RPP) confers resistance upon the microorganism via protection of the site of bacteriostatic antibiotic action (high Tet-affinity sites on the 30S ribosomal subunit). This phenomenon was first reported by Burdett (1986), based on work with *Streptococci*. The second mechanism of resistance relies on the reduction of antibiotic concentrations within the microbial cell. This is achieved by energy-dependent transport of the antibiotic from within the cytoplasm by efflux pumps (Schanappinger and Hillen, 1996). The active excretion of TC from the cell may occur via a multidrug resistance pump (Li et al., 1995) or by TC-specific transporters in both gram-negative and gram-positive bacteria (Schanappinger and Hillen, 1996). The enzymatic inactivation of the antimicrobial agent within the cell is the third mechanism for antibiotic resistance, with the TetX determinant identified in *Bacteroides* (Speer et al., 1991).

Considerable research has been devoted towards studying the fate of antibiotics and other pharmaceutical compounds that are excreted by farm animals (Chiu et al., 1990; Cohen, 1998; Elmund et al., 1971; Kelley et al., 1998; Mathew et al., 1998; Wade and Barkley, 1992). Nevertheless, most previous studies involving antibiotic resistance detection have focused on isolation and identification of resistant strains in field samples from sites of antibiotic application. Currently, little is known about how such gene reservoirs are developed, maintained, or amplified. This suggests the need for a better understanding of the fate, transfer, and natural attenuation of pertinent genetic determinants when released to the environment during direct runoff, groundwater infiltration from lagoons, or manure spreading activities. Critical knowledge gaps include the rate and extent of resistance-gene propagation (including bacterial migration and interspecific gene transfer from enteric to soil bacteria), and the effect of environmental factors and exposure conditions on the persistence of bacterial antibiotic resistance.

This paper reports the isolation and identification of Tet^r soil bacteria and their genetic determinants. Emphasis was placed on monitoring total heterotrophic and Tet^r bacterial concentrations to characterize phenotypic shifts under the selective pressure exerted by TC. The recovery of the microbial community upon TC removal was also monitored.

2. Materials and methods

2.1. General approach

Flow-through columns packed with soil were used to mimic runoff and infiltration of TC-contaminated agricultural drainage, and to evaluate changes in the total heterotrophic and Tet^r microbial populations during and after sustained TC exposure. Acetate, which is a common product of animal waste breakdown and is likely to be present in farm runoff, was added as a carbon source. A control column without TC was also run. Emphasis was placed on enumerating and characterizing bacteria in the column effluent to focus on mobile bacteria with a higher potential to reach a human recipient. Along with population enumerations, TC and acetate concentration profiles were monitored along the length of the columns to investigate TC stability and acetate utilization patterns. Tet^r microorganisms isolated from the effluent of the TC-enriched column were identified by genetic analysis and screened for the tet-determinants responsible for TC resistance. The recovery of the microbial populations after TC exposure ceased was also characterized by monitoring the percentage of Tet^r heterotrophs in the column effluent.

2.2. Flow-through columns

Two flow-through glass columns (30-cm long, 2.5-cm inner diameter) (Kontes Glass Company, Vineland, NJ) were equipped with six sampling ports located at 2, 5, 9, 14, 19 and 24 cm from the bottom inlet of the column. The sampling ports had inlet/outlet three-way valves (Hamilton Co.). The columns were secured in a vertical position and packed with soil (University of Iowa Softball Field, 22% sand, 74% silt, 4% clay, $f_{oc} = 0.056$) as described elsewhere (Alvarez et al., 1998) to preclude trapping air bubbles. The columns were wrapped in aluminum foil to minimize algal growth and antibiotic photodegradation. The feed reservoirs were 2-L bottles equipped with 3-hole caps (Kontes Glass Company, Vineland, NJ) and wrapped in aluminum foil. Masterflex Neoprene® tubing (Cole-Parmer Co.) and a Masterflex peristaltic pump (Cole-Parmer Co.) were used to deliver the feed solution (pH = 7.2 for control column, pH = 6.2 for TC-enriched column (lower due to acidity of TC solution), $T = 25\,^{\circ}C$). The flow rate was about $3.5\,mL\,h^{-1}$, corresponding to a Darcy velocity of 0.7 cm/day. Bromide tracer studies indicated a porosity of 0.36 and a hydraulic retention time of about 15 h. The feed solution for the control column consisted of bicarbonate buffered, synthetic ground water (von Gunten and Zorbist, 1993) amended with sodium acetate as a carbon source ($10\,mg\,L^{-1}$). TC (T3383, Sigma Co.) was also added continuously (at $50\,mg\,L^{-1}$) to the influent of the amended column. This

TC concentration is higher than typical background levels found in the environment ($\sim 1\,\mu g/L^{-1}$) (Boxall et al., 2003), but it may be representative of concentrations in or around animal farms and it is sufficiently high to select for resistant bacteria (Aminov et al., 2001; Chee-Sanford et al., 2001).

2.3. Chemical analyses

Acetate and TC concentrations were monitored bimonthly along the length of the columns. Acetate was measured with an ion chromatograph equipped with an auto-sampler (Alltech 570), an IonPAc AS14 column (Dionex), and a conductivity detector (Dionex). TC was analyzed by HPLC (Alltech 426), using a C859353-U column (Supelco) and a variable wavelength detector (Dionex). Detection conditions were as follows: 680 mL 0.1 M ammonium oxalate, 270 mL dimethylformamide, 50 mL 0.2 M dibasic ammonium phosphate (pH 7.6), at $1\,mL\,min^{-1}$, 20 μL, Isocratic, Ambient, UV at 280 nm.

2.4. Batch studies

The stability of TC in contact with soil was studied in 250-mL serum bottles filled with 100-mL of distilled water and 10 g of soil ($pH \approx 7$). A $100\,mg\,L^{-1}$ TC solution was prepared ($pH \approx 4$) and 50 mL were added to the soil mixture. The pH and the TC concentrations were measured within 1 min after TC addition. Heat-sterilized controls were also prepared to assure that microbial activity was not responsible for TC degradation.

2.5. Microbial enumeration

Total heterotrophic and Tet^r bacteria concentrations were measured periodically in the effluent of the columns to study the effect of sustained TC exposure on the development of antibiotic resistance. Microbial enumerations were also conducted after ceasing TC exposure to characterize the recovery response of the microbial community in the absence of selective pressure by TC. A modified Most Probable Number (MPN) 96-well plate technique was used for microbial enumeration (Lovanh et al., 2002). Tryptic soy broth (TSB, Difco Laboratories) was used to enumerate total heterotrophs, and TSB enriched with TC ($50\,mg\,L^{-1}$) was used to enumerate Tet^r bacteria. Whereas TSB is a nutrient-rich medium that might not be suitable to grow oligotrophic soil bacteria (Atlas and Bartha, 1998), it is well suited to enumerate r-strategists that prevail in nutrient-rich environments, such as soil impacted by animal farm runoff or the bio-stimulated (nutrient- and acetate-enriched) columns that we used. Bacterial quantification was based on visual scoring of growth-induced TSB-turbidity development within the well plates, and subsequent statistical analysis, based on the Poisson distribution (Metcalf and Eddy, 1991). All analyses were conducted in triplicate samples.

2.6. Genetic analysis

Effluent from the TC-exposed column was used for the isolation of antibiotic-resistant strains. TC-enriched ($50\,mg\,L^{-1}$) R2A agar plates (Difco Laboratories) were streaked with column effluent (0.1 mL) and incubated at 30 °C, 2–5 days, depending on the growth rates (appearance of colonies). Individual colonies were re-streaked onto TC-enriched R2A agar plates, incubated, isolated and re-streaked a second time in order to ensure strain "purity".

Bacterial DNA was extracted from selected colonies with kits according to manufacturers protocols (Qiagen). A Mastercycler® thermocycler device (Eppendorf) was used for the polymerase chain reaction (PCR) gene detection techniques. PCR amplification was performed on the extracted DNA according to the protocols provided in the reaction kits (PanVera). The final concentrations of the PCR reagents in a 50 μL reaction mixture were: 1.25 U DNA polymerase (Ex Taq), 1X reaction buffer, 200 μM doexynucleoside triphosphate, and 0.2 μM primers (forward and reverse). Primers were constructed (according to Table 1) for the following tet-determinants coding for ribosomal protection proteins (RPP): TetB(P), Tet(M), Tet(O), Tet(Q), Tet(S), Tet(T), Tet(W), and OtrA. The amplification was performed as previously described by Aminov et al. (2001). Briefly, the cycle steps were: (1) an initial denaturation at 94 °C (5 min) followed by 25 cycles at 94 °C (30 s), (2) annealing at 30 s, and 30 s extension (72 °C), and (3) extension at 72 °C (7 min). The annealing temperatures for each primer are shown in Table 1. Reaction products were analyzed by electrophoresis on a 1.2% (wt/vol) agarose gel containing ethidium bromide.

Primers targeting efflux-pump tet-determinants were constructed according to Table 2. Amplification conditions were as described by Furushita et al. (2003), and included 30 cycles of 60 s at 94 °C, 45 s at annealing temperatures shown in Table 2, and 90 s at 72 °C, followed by a final extension of 300 s at 72 °C. PCR reaction products were purified with the QIAquick® PCR Purification Kit (Qiagen) according to manufacturer's protocol. Restriction digests of the PCR products were attempted with the following endonucleases (to identify the following *tet* determinants): *SmaI* (TetA), *SphI* (TetB, TetD, TetY), *SalI* (TetC), *NdeII* (TetE, TetH, TetJ), and *EcoRI* (TetG). The amplicons were analyzed by gel-electrophoresis as described above.

Escherichia coli reference strains containing the Tet^r determinants listed in Table 1 were donated by Svetlana Kocherginskaya and Rod Mackie (University of Illinois

Table 1
PCR primers targeting ribosomal protection protein (RPP) classes

Tet-determinant targeted	Primer sequence	Amplicon size (bp)	Annealing temperature (°C)
TetBP-F	AAAACTTATTATATTATAGTC	169	46
TetBP-R	TGGAGTATCAATAATATTCAC		
TetM-F	ACAGAAAGCTTATTATATAAC	171	55
TetM-R	TGGCGTGTCTATGATGTTCAC		
TetO-F	ACGGARAGTTTATTGTATACC	171	60
TetO-R	TGGCGTATCTATAATGTTGAC		
OtrA-F	GGCATYCTGGCCCACGT	212	66
OtrA-R	CCCGGGGTGTCGTASAGG		
TetQ-F	AGAATCTGCTGTTTGCCAGTG	169	63
TetQ-R	CGGAGTGTCAATGATATTGCA		
TetS-F	GAAAGCTTACTATACAGTAGC	169	50
TetS-R	AGGAGTATCTACAATATTTAC		
TetT-F	AAGGTTTATTATATAAAAGTG	169	46
TetT-R	AGGTGTATCTATGATATTTAC		
TetW-F	GAGAGCCTGCTATATGCCAGC	168	64
TetW-R	GGGCGTATCCACAATGTTAAC		

Source: Aminov et al. (2001).

Table 2
PCR primers targeting efflux-pump determinants

Tet-determinant targeted	Primer sequence	Amplicon size (bp)	Annealing temperature (°C)
TetA-F	CGCYTATATYGCCGAYATCAC	417	55
TetA-R	CCRAAWKCGGCWAGCGA		
TetB-F	GGDATTGGBCTTATYATGCC	967	50
TetB-R	ATMACKCCCTGYAATGCA		
TetC-F	CGCYTATATYGCCGAYATCAC	417	55
TetC-R	CCRAAWKCGGCWAGCGA		
TetD-F	GGDATTGGBCTTATYATGCC	964	50
TetD-R	ATMACKCCCTGYAATGCA		
TetE-F	GGDATTGGBCTTATYATGCC	650	50
TetE-R	AWDGTGGCDGGAATTTG		
TetG-F	TATGCRTTKATGCAGGTC	917	50
TetG-R	GACRAKCCAAACCCAACC		
TetH-F	GGDATTGGBCTTATYATGCC	650	50
TetH-R	AWDGTGGCDGGAATTTG		
TetJ-F	GGDATTGGBCTTATYATGCC	650	50
TetJ-R	AWDGTGGCDGGAATTTG		
TetY-F	TATGCRTTKATGCAGGTC	911	50
TetY-R	GACRAKCCAAACCCAACC		

Source: Furushita et al. (2003).

at Urbana Champaign), and were used as positive controls for PCR method validation purposes. Tet^r bacteria isolated from the column effluents were identified based on their 16S rRNA sequence by Microbial Insights, Inc. (Rockford, Tenn), using the BLASTN of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov).

3. Results and discussion

This study investigated the effect of a widely used antimicrobial agent on the phenotypic response of indigenous soil microorganisms in parallel with the identification of genetic elements responsible for antibiotic resistance in Tet^r isolates. Flow-through columns were used to mimic runoff and infiltration around

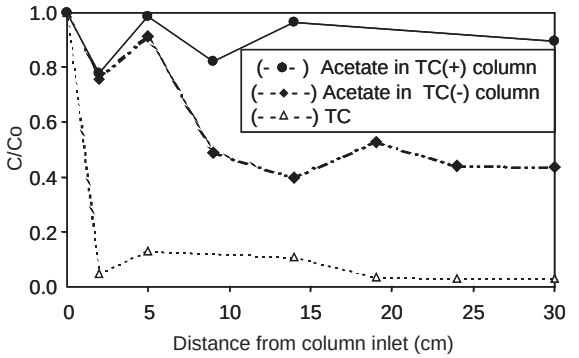

Fig. 1. Acetate and TC concentration profiles after 60 days of operation with a hydraulic residence time of 15 h. The data show decreased heterotrophic activity (less acetate consumption) for the TC-enriched and column compared to the control. TC removal (possibly due to hydrolysis and sorption) occurred near the inlet, presumably upon contact with the soil.

agricultural sites that administer TC for disease prevention and animal growth promotion.

Acetate concentration profiles (Fig. 1) were monitored along the length of the columns to evaluate microbial utilization of the carbon source (acetate) as an indication of heterotrophic activity. Acetate concentrations reflect the bacteriostatic effect of TC, which significantly decreased microbial heterotrophic activity. Only about 10% of the influent acetate was utilized in the TC-amended column, compared to about 50% utilization for the control column without TC. The lack of acetate utilization in the downstream segment of the TC-free control column (8–30 cm, Fig. 1) was likely due to dissolved oxygen depletion, which would have precluded aerobic metabolism of acetate. Profiles taken one year later showed nearly complete acetate removal from this column, presumably due to anaerobic degradation, which typically requires longer acclimation time (data not shown).

A significant decrease in the aqueous TC concentration occurred near the inlet, and only trace amounts of TC (approximately 4% of influent concentration) were detectable throughout the length of the column. TC removal was presumably due to abiotic degradation upon contact with soil under neutral or mild alkaline conditions (pH = 6.5–9.0) (Halling-Sorensen et al., 2002). Furthermore, although TC is very soluble in water ($S = 1700\,\text{mg/L}$, $\log k_{ow} = -1.19$), sorption by other mechanisms than hydrophobic partitioning (e.g., cation bridging at clay surfaces and surface complexation) probably contributed to TC removal from the aqueous phase (Tolls, 2001). It is unlikely that TC removal was due to microbial degradation because very fast TC removal (96% within 1 min) was observed in batch studies where TC (100 mg/L, pH = 4) was added to soil slurries (pH = 7) (data not shown).

TC exerted a significant bacteriostatic effect, decreasing the MPN concentration of total heterotrophs eluting from the TC-amended column by one order of magnitude compared to the control (Fig. 2A). Nevertheless, the effluent concentration of Tet^r bacteria was significantly higher for the TC-amended column than for the control ($p < 0.05$) (Fig. 2B). Thus, TC exerted selective pressure for the development and maintenance of antibiotic resistance in soil bacteria, even though, to the best of our knowledge, potential Tet^r gene donors such as enteric Tet^r bacteria that could be excreted by farm animals were not initially present in this soil. Whereas TC concentrations decreased rapidly near the inlet, some TC degradation products such as 5a-6-anhydrotetracycline and 5a,6-anhydrochlorotetracycline (none of which were examined) are known to retain

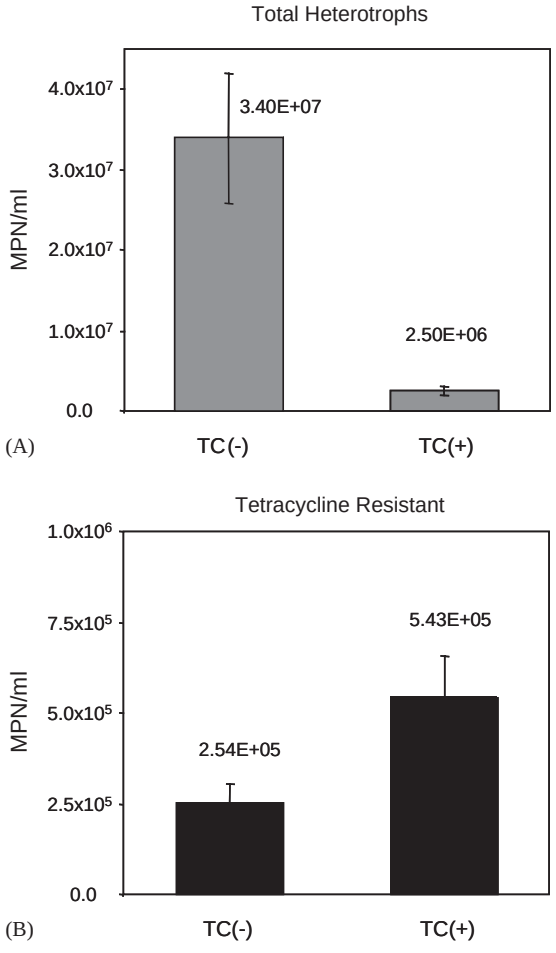

Fig. 2. Effluent microbial concentrations from two columns. The TC(+) column was exposed to tetracycline during 300 days, whereas the TC(–) column served as an unexposed control. The concentration of total heterotrophs was significantly lower in the TC(+) column, while Tet^r bacteria concentrations were significantly higher ($p < 0.05$) (B).

antimicrobial properties (Halling-Sorensen et al., 2002). Thus, it is plausible that TC degradation products, which may result from abiotic reactions, also contributed to the selective pressure for Tet^r bacteria.

TC addition to the treated column was stopped after 300 days. Following a 2-week lag, this resulted in a significant decrease in the percentage of Tet^r heterotrophs, from about 25% after sustained TC exposure to the pre-exposure and control levels (1–2%) within 30 days (Fig. 3). This trend was due both to a rebound of total heterotrophs (with a related increase in acetate consumption) as well as to a significant decrease in Tet^r bacteria concentration (data not shown). It should be pointed out that the concentration of total heterotrophs eluting from the column previously enriched with TC did not reach the same levels eluting from the control column within the 30-day monitoring period, possibly due to some residual antibiotic activity. Nevertheless, Fig. 3 suggests that discontinuing TC exposure or curtailing its use should enhance natural attenuation mechanisms that mitigate the spread of resistance vectors.

Tet^r bacteria were isolated from the TC-enriched column effluent by plating on Tet-R2A agar. Such isolates represent mobile bacteria that could reach human recipients. Two types of Tet^r microbial colonies were consistently detected and isolated. The first isolate was identified on the basis of its 16S rRNA sequence (using the BLAST database) as *Burkholderia cepacia*, which is a common soil bacterium. The second isolate was identified on the basis of its 26S rRNA sequence (using the NTBI database) as *Rhodotorula mucilaginosa*, which is a fungus. The identity of this yeast was confirmed by analysis with an API 20 C AUX yeast system kit.

Burholderia cepacia was screened for 17 tet-determinants coding for both ribosomal protection proteins (Table 1) and efflux pumps (Table 2), and it was found to carry an efflux pump gene (TetA or TetC) (Fig. 4). Discerning whether the determinant was TetA or Tet C was not possible because the same primer was used for both genes (Table 2) and restriction digest attempts of the amplicon did not result in detectable fragments. Attempts to amplify TetA and TetC sequences using separate primers described by Aminov et al. (2002) were unsuccessful, suggesting that the target Tet^r sequence for this *B. cepacia* strain may be slightly different than the sequences used by Aminov et al. (2002).

The development of TC resistance in soil bacteria exposed to TC suggests that indigenous soil microorganisms may serve as reservoirs for the propagation (and possibly the amplification) of antibiotic resistance, and potentially pose a direct hazard to public health. *B. cepacia* has been a focus of attention due to its (opportunistic) pathogenic characteristics (Kiska et al., 1996, Govan et al., 1996). Yet, *B. cepacia* has also received considerable attention due to its broad substrate specificity for application in bioremediation processes (Bourquin et al., 1997, Steffan et al., 1999). This work suggests that the potential for disease propagation by *B. cepacia* due to acquired antibiotic resistance should be considered in its selection for bioaugmentation applications.

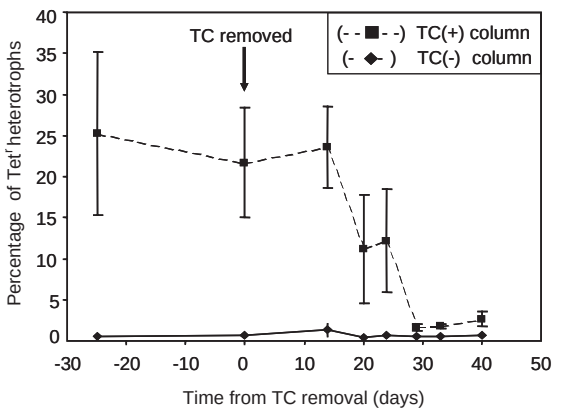

Fig. 3. Decrease in the percentage of Tet^r heterotrophs after TC removal from column feed solution.

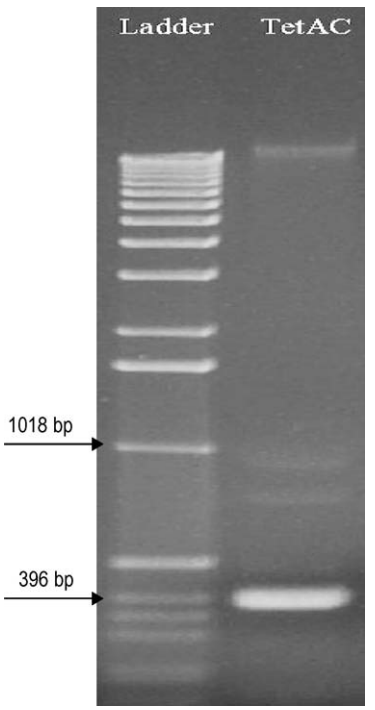

Fig. 4. PCR amplification of the TetA/C gene determinant of *B. cepacia*, coding for an efflux pump. Amplicon size of approximately 417 bp (as reported by Furushita et al. (2003) along with 1 Kb ladder are presented.

Interestingly, the fungal microorganism (*R. mucilaginosa*) was only detected on R2A plates during the TC-enrichment period. This yeast was never detected in the effluent from the control column without TC or in the treated column effluent after TC exposure ceased. Since yeasts are relatively insensitive to TC (i.e., the TC mode of action involves the inhibition of prokaryotic protein synthesis by preventing the association of aminoacyl-tRNA with the 30S bacterial ribosome subunit that is not present in the eukaryotic fungi) (Chopra and Roberts, 2001), we speculate that *R. mucilognosa* proliferation during TC exposure was due to the inhibition (or death) of bacteria that were antagonistic to this yeast. This implies that TC exposure might affect microbial community structure not only through its direct bacteriostatic effect, but also indirectly by influencing microbial interactions among different populations.

4. Summary and conclusions

This study investigated the phenotypic response of soil microbial communities exposed to selective pressure by tetracycline. Sustained exposure to TC resulted in a significant increase in the concentration of tetracycline-resistant soil bacteria, as well as a large decrease in the concentration of total heterotrophs. This suggests that TC release to the environment by animal agriculture is conducive to the development and amplification of antibiotic resistance, with soil bacteria serving as resistance reservoirs for Tet^r continuance. Nevertheless, removing the selective pressure by TC resulted in phenotypic shifts that returned the microbial community to initial conditions within 1 month, which implies that discontinuing TC exposure or curtailing its use should enhance natural attenuation mechanisms that mitigate the spread of resistance vectors.

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