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PCR Detection of Oxytetracycline Resistance Genes *otr*(A) and *otr*(B) in Tetracycline-Resistant Streptomycete Isolates from Diverse Habitats

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Abstract. A range of European habitats was screened by PCR for detection of the oxytetracycline resistance genes *otr*(A) and *otr*(B), found in the oxytetracycline-producing strain *Streptomyces rimosus*. Primers were developed to detect these *otr* genes in tetracycline-resistant (Tc^R) streptomycete isolates from environmental samples. Samples were obtained from bulk and rhizosphere soil, manure, activated sludge and seawater. The majority of Tc^R streptomycetes originated from bulk and rhizosphere soil. Fewer Tc^R streptomycetes were isolated from manure and seawater and none from sewage. By PCR, three out of 217 isolates were shown to contain the *otr*(A) gene and 13 out of 217 the *otr*(B) gene. Surprisingly, these genes were detected in taxonomic groups not known as tetracycline-producing strains. The majority of the *otr* gene–carrying strains was assigned to *S. exfoliatus* or *S. rochei* and originated from all habitats from which Tc^R streptomycetes were obtained. Our results indicated that the occurrence of *otr*(A) and *otr*(B) genes in natural environments was limited and that *otr*(B), in comparison to *otr*(A), seemed to be more common.

Keywords: tetracycline resistance genes, antibiotics, *otr*(A), *otr*(B), streptomycetes, PCR detection.

Tetracyclines are clinically important drugs and the development and spread of resistance remains a major concern. Of particular interest are the natural reservoirs of such resistance genes. Recent studies have attempted to evaluate the impact of tetracycline use on communities of Gram-negative [1, 14] and Gram-positive bacteria [2, 22] in various natural environments. Reports on the presence of tetracycline resistance genes in streptomycetes are limited to the producer strains, such as *Streptomyces rimosus* [3] and *S. aureofaciens* [4], selected non-producing species such as *Streptomyces lividans* 1326 [5] and pathogenic actinomycetes with clinical importance, such as *Mycobacterium fortuitum* and *M. peregrinum*, as well as pathogenic streptomycetes [18]. So far, tetracycline resistance in naturally occurring streptomycetes has not been studied.

In this study, we assessed the prevalence of the two oxytetracycline resistance genes, *otr*(A) and *otr*(B), found on the chromosome of oxytetracycline producer *Streptomyces rimosus*, in streptomycete populations of environmental samples [7, 16]. The habitats selected represented sites with either a history of pollution or antibiotic selective pressure or more pristine environments and comprised of bulk and rhizosphere soil, seawater, manure, and sewage. In a comprehensive survey of the occurrence of antibiotic resistance genes and their mobility, the same samples were analysed using a multiphasic approach for antibiotic resistance genes typically found in non-producing bacteria [12, 24].

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Table 1. Streptomycetes and plasmids used in this study

Strains and plasmids	Resistance gene	Resistance mechanism	Tetracycline production
S. rimosus ATTC 10970	*otr*(A)	Ribosomal protection	Oxytctracycline
S. rimosus ATTC 10970	*otr*(B)	Efflux	Oxytetracycline
S. lividan 1326	*tet*	Ribosomal protection	–
S. aureofaciens NRRL3203	*tcr3*	Efflux	Chlortetracycline
pFD310	*tet*(M)	Ribosomal protection	–
pAT451	*tet*(O)	Ribosomal protection	–
RP1	*tet*(A)	Efflux	–
pBR322	*tet*(C)	Efflux	–
PSL1504	*tet*(E)	Efflux	–

Table 2. Origin of environmental samples that were studied

Type of sample	Description and origin of sample (Sample No)
Bulk soil	Dossenheim (Germany); plantomycin (active compound streptomycin sulphate) treated soil (1)
	Dossenheim; untreated soil (2)
	Drotwichn (UK), sewage sludge treated soil (3)
	Costwolds (UK); limestone-based soil fallow (sparse vegetative cover) (4)
Rhizosphere soil	Dossenheim, grass grown on plantomycin (active compound streptomycin) treated soil (5)
	Dossenheim; grass grown on untreated soil (6)
	Ens (The Netherlands); white radish (*Raphanus sativus* L. rettich) grown on $CuSO_4$ treated soil (7)
	Ens; white radish *(R. sativus* L. rettich) grown on untreated soil (8)
Manure	Broiler chicken grown on flavomycin-treatcd food (Germany) (9)
	Broiler chicken grown on untreated food (Germany) (10)
	Layer chicken grown on Zn-bacitracin-treated food (Germany) (11)
	Layer chicken grown on untreated food (Germany) (12)
Activated sludge	Brussels; Hospital wastewater treatment facility (Erasmus Hospital; Belgium) (13)
	Ghent; Hospital wastewater treatment facility (Maria Middelares Hospital; Belgium) (14)
	Wavre; treatment plant of the Dyle valley (population, industry, university; Belgium) (15)
	Rosière; treatment plant of Lasne (population, industry, Belgium) (16)
Seawater	Athens; wastewater treatment outflow in Saronicos Gulf (Greece) (17)
	Volos, Pagasiticos Gulf; fishfarm tetracycline administered at regular base (Greece) (18)
	Eretria (Evia Island): pristine (Greece) (19)
	Fleves Island; pristine (Greece) (20)

Materials and Methods

Bacterial strains and culture conditions. Streptomycetes and *E. coli* strains carrying tetracycline resistance genes were used in this study (Table 1). These strains were grown on Tryptic Soy Agar (TSA, Oxoid) and Luria Agar (Oxoid), respectively, amended with 50 μg mL^{-1} of tetracycline at 27°C. Strains of *S. griseus, S. coelicolor* and *S. fradiae* with phenotypes sensitive to tetracycline were also employed and were grown on TSA at 27°C.

Sampling and selective isolation of Tc^R streptomycetes. Twenty European sampling sites were selected and samples were taken from bulk soil, rhizosphere soil, manure, activated sludge and seawater (Table 2). The sampling sites and the sample processing methodology have been described in detail by Heuer et al. [12] and van Overbeek et al. [24]. Sampling from four different locations for each habitat gave the opportunity to include two samples from habitats under selective pressure, for example, pollution, heavy metals or antibiotic treatments and two samples from sites that were not known to be receiving treatments. Bacterial suspensions of all samples were placed in a water bath for 2 h at 60°C to eliminate the growth of other bacterial groups and to enhance the possibility of heat-tolerant streptomycetes to grow. Serial dilutions (10^0 to 10^{-6}) were plated on the streptomycete selective media RASS and AGS [9], supplemented with 10 μg mL^{-1} tetracycline and 100 μg mL^{-1} cycloheximide, to inhibit fungal growth and incubated at 28°C for 3 to 10 days. Colonies with different color and morphology (maximally 24 individual colonies from each sample) were purifired and spore suspensions of isolates were stored in 20% glycerol solution at −70°C [13].

Denaturing Gradient Gel Electrophoresis (DGGE). The 16S rRNA gene–based PCR-DGGE method, as described by Heuer et al. [11], was applied to all Tc^R streptomycete isolates obtained. For this purpose, genomic DNA was extracted from streptomycete isolates, using a protocol described by Hopwood et al. [13] and subjected to amplification using the actinomycete specific primers F243 and R513gc [11]. Amplicon profiles from all isolates were used in order to cluster isolates into groups and reduce the number of isolates used for further identification analysis, by selecting only representatives from each one of the assigned groups.

Characterization of culturable resistant phenotypes. Identification of isolates was carried out using 41 morphological and physiological diagnostic characters for phenotypic identification [15]. Isolates were

identified using the probabilistic identification matrix of Williams et al. [25]. In this scheme, three identification statistics were used: the Wilcox probability, taxonomic distance and its standard deviation [25].

Primer design and PCR detection of *otr* genes. PCR technique was employed to detect *otr*(A) and *otr*(B) gene sequences among the Tc^R streptomycetes isolated in this study. Selected PCR primer sets were tested for amplification of their respective target genes. They were also assessed for the absence of any PCR product when targeting tetracycline-sensitive streptomycete DNA and plasmid DNA containing tetracycline resistance genes (Table 1), non-homologous to *otr* genes. Genomic DNA was extracted from streptomycete isolates, as described by Hopwood et al. [13] and plasmid DNA was extracted from the Tc^R *E. coli* reference strains according to a standard protocol [21]. PCR amplification was performed in a Thermal Cycler (Genius, Techne, Cambridge, UK), and the reaction mixture was as follows: Tris-HCl pH 8.3, 50 mM; KCl 50 mM; $MgCl_2$ 2.5 mM; 0.1 mg mL^{-1} bovine serum albumin (Sigma); 200 μM of each deoxynucleoside triphosphate; 2.5 % dimethyl sulfoxide; 100 nM of each oligo; 1 U of TaqPolymerase (Promega, Madison, WI) and 1 μL (ca. 20 ng) of template DNA. Amplified fragments were resolved by agarose gel (1%) electrophoresis in TBE buffer followed by ethidium bromide staining.

Southern blot analysis of *otr*(A) and *otr*(B). PCR-amplified *otr*(A) and *otr*(B) fragments were confirmed by Southern blot assays [10]. Amplicons in agarose gels were transferred onto nylon membranes (Nytran SuperCharge, Schleicher & Schuell, Germany). Membranes were hybridized by digoxigenin-labelled DNA probes (Roche, Mannheim, Germany) generated by PCR amplification of *otr*(A) and *otr*(B) from *S. rimosus* ATTC 10970. Southern hybridization signals of PCR products were detected using the DIG detection kit (Roche).

Cloning and sequencing. PCR products obtained with *otr*(A) and *otr*(B) primers were ligated into the pCR 2.1 TOPO vector according to the instructions of the manufacturer (Invitrogen, La Jolla, CA). After transformation of TOPO10F competent cells (Invitrogen), clones were picked and the presence of inserts of the expected size was assessed. Selected clones were then sequenced with vector-specific primers on a PE-ABI377 sequencer (IMBB, Crete, Greece).

Results

Characterization of Tc^R streptomycete isolates. From all environmental samples, 217 Tc^R *Streptomyces* colonies were isolated. Out of these isolates, 66% originated from bulk soil, 9.6% were isolates from rhizosphere soil samples, 16% were from manure and 7.3% from seawater samples. Interestingly, no streptomycete growth was observed on plates inoculated with activated sludge samples. All isolates were screened by analysis of 16S rRNA gene amplicons by denaturing gradient gel electrophoresis. The band position of isolates was examined and strains were assigned to seven migration groups based on the final position in the gel (Fig. 1). Groups were not unique for the samples. Four of the seven groups had isolates originating from different samples. Randomly, ten representatives from each of the groups (where a group contained more than ten isolates) were selected and were further characterized phenotypically. Identification results of all representatives from each group were alike. This analysis revealed the presence of 58 *S. exfoliatus* isolates (group A), 14 *S. albidoflavus* (group B) and 30 *S. viridosporus* isolates (group C) and 42 isolates that could not be identified (Group D) in bulk soil samples. Seven isolates originating from the rhizosphere soil samples belonged to *S. rochei* cluster (group E) and 14 isolates to *S. albidoflavus* cluster (group B). Isolates of group F, which were identified as *S. collinus* strains, were found in both manure (21 isolates) and seawater (five isolates) samples, whilst 15 manure isolates belonging to group A were characterized as *S. exfoliatus* strains. Eight of the marine Tc^R streptomycete strains were identified as *S. rochei* (group E) and there were three marine isolates (group G) that could not be identified.

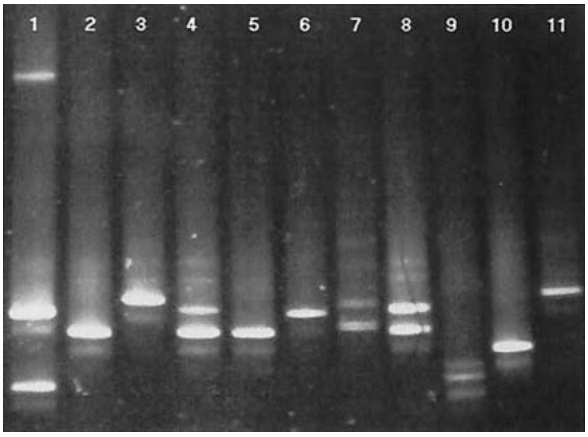

Fig. 1. DGGE analysis of 16S rRNA gene amplicons from Tc^R streptomycete isolates. Lanes 2, 4, 5, 8: amplicons from strains that belong to group B; lanes 1, 3, 6, 7, 9, 10, 11: amplicons from strains of groups A, C, D, E, F, G respectively.

Design and validation of *otr*(A) and *otr*(B) primers. Primers were designed for the detection of *otr*(A) and *otr*(B)-like genes in streptomycetes (Table 3). The rationale of primer design was to specifically target the *otr*(A) and *otr*(B) genes found in *S. rimosus*, but also to amplify the homologous to *otr*(A) tetracycline resistance gene *tet* found in the non-producer *S. lividans* and the *trc*3 gene found in the chlortetracycline producer *S. aureofaciens*, which is homologous to *otr*(B) (Table 1). Control PCR tests were performed with *E. coli* strains carrying plasmids with other known Tc^R genes, non-homologous to *otr* genes. The absence of signals indicated that the selected primers did not amplify Tc^R genes distant to the *otr* genes found in streptomycete strains. PCR products, of 778 bp for the *otr*(A) amplicon and 947 bp for the *otr*(B) amplicon, both amplified from *S. rimosus* were cloned and sequenced, to verify the specificity of the primers. Alignment of sequences

Table 3. PCR primers, cycles of amplification, primer position and sizes of amplified DNA fragments of this study

Oligo name	Primer sequence 5′-3′	Cycle of amplification	Amplicon (bp)	Primer position	Origin[a]
otr(A) (F)[b]	GAACACGTACTGACCGAGAAG	4 min/94°C, 35 × (1 min/94°C, 1 min/55°C, 2 min/72°C) and 10 min/72°C	778	604	X53401/*S. rimosus*
otr(A) (R)[c]	CAGAAGTAGTTGTGCGTCCG			1730	ATTC 10970
otr(B) (F)	CCGACATCTACGGGCGCAAGC	4 min/94°C, 35 × (1 min/94°C, 1 min/55°C, 2 min/72°C) and 10 min/72°C	947	251	AF079900/*S. rimosus*
otr(B) (R)	GGTGATGACGGTCTGGGACAG			1177	ATTC 10970

[a] Genbank accession number and strain on which primers were based.
[b] Forward primer.
[c] Reverse primer.

Table 4. Streptomycete hosts of *otr* genes isolated from different environmental samples

Detected gene	Isolate	Identity (cluster)[a]	Wilcox probability	Taxonomic distance	Standard error	Sample No.
otr(A)	WIC2	*S. exfoliatus*	1.000	0.382	1.178	1
otr(A)	W2B6	*S. viridosporus*	0.902	0.802	0.976	2
otr(A), *otr*(B)	ER2	*S. rochei*	0.978	0.415	2.055	19
otr(B)	W2A5	*S. viridosporus*	0.875	0.452	1.436	2
otr(B)	W2D2	*S. viridosporus*	0.891	0.638	0.821	2
otr(B)	W4B3	*S. exfoliatus*	0.993	0.390	1.381	4
otr(B)	W4B4	*S. exfoliatus*	0.939	0.674	1.648	4
otr(B)	W4B1	*S. albidoflavus*	0.991	0.452	2.125	4
otr(B)	ENS4	*S. rochei*	0.943	0.485	0.976	8
otr(B)	ENS10	*S. rochei*	0.892	0.442	1.102	8
otr(B)	ENS11	*S. rochei*	0.921	0.878	1.436	8
otr(B)	B1	*S. exfoliatus*	0.934	0.514	0.584	9
otr(B)	B4	*S. exfoliatus*	0.945	0.362	0.481	9
otr(B)	B7	*S. exfoliatus*	0.974	0.784	1.508	9
otr(B)	FF2	*S. rochei*	0.918	0.647	1.005	18

showed 100% similarity to the published sequences of the respective genes from *S. rimosus*. No amplification product was observed in PCR tests with chromosomal DNA from tetracycline-sensitive streptomycete strains such as *S. griseus, S. coelicolor* and *S. fradiae*.

Distribution of *otr* determinants within streptomycete isolates. All 217 Tc^R streptomycetes were analyzed for the presence of *otr* genes using PCR followed by hybridization with the appropriate probe. Only 15 isolates, obtained from dilution plates not higher than 10^{-2}, showed signals with any one of the probes, and one (*S. rochei* ER2 of marine origin) gave positive hybridization with both probes. The *otr*(A) gene was detected in two isolates from the Dossenheim soil samples identified as *S. exfoliatus* and *S. viridosporus*, and in one *S. rochei* strain from the seawater sample from Evia Island (Table 4). None of the rhizosphere soil or manure isolates carried *otr*(A). The *otr*(B) gene was detected in thirteen isolates: five *S. exfoliatus* from Cotswold soil and antibiotic treated broiler chicken manure, five *S. rochei* isolated from rhizosphere soil (untreated Ens site) and seawater (fishfarm and Evia Island), two *S. viridosporus* isolates from Dossenheim soil and one *S. albidoflavus* from Cotswold soil and (Table 4). The presence of the *otr* genes was confirmed by sequencing the amplicons obtained from isolates. Comparison of these sequences showed that all three *otr*(A)-amplicons and twelve *otr*(B)-amplicons were 100% identical with the respective sequenced *otr* genes *S. rimosus*, whereas two *otr*(B)-amplicons showed 92% similarity to the *S. rimosus otr*(B) gene (Table 4).

Discussion

This work is, to the best of our knowledge, the first attempt to study tetracycline resistance and to estimate

the gene pool and flux of resistance genes in environmental streptomycetes. Tc^R streptomycetes were found in all samples, with the exception of sewage that provided no isolates. Although the taxonomic composition varied among the environmental samples, only seven different streptomycete groups were found based on DGGE analysis. An interesting observation was the absence of tetracycline-producing strains, such as *S. rimosus* and *S. aureofaciens*, among the Tc^R isolates. This is in contrast with the findings of Egan and coworkers [8] and Tolba and co-workers [23]. They showed the majority of the streptomycin-resistant streptomycetes isolated from soil samples were identified as *S. griseus*, a streptomycin-producing strain.

The present study indicated that only a small proportion (~7%) of the streptomycete isolates screened, with phenotypic resistance to tetracycline, contained *otr*(A) or *otr*(B). This is not the only case that phenotypically Tc^R isolates did not hybridize with any of the *tet* probes studied [3]. This could imply that some of the isolates may carry *tet* genes that were not screened in this study. For example, *tet*(K) and *tet*(L) genes are widely distributed among Gram-positive species and have been found in *Mycobacterium, Nocardia* and *Streptomyces* spp. isolated from humans [6, 18]. However it is shown that not all Tc^R Gram-positive bacteria have been reported to carry specific known *tet* genes [20].

The isolates that contained *otr* genes were taxonomically grouped within a range of four streptomycete taxa: *S. exfoliatus*, *S. rochei*, *S. viridosporus* and *S. albidoflavus*, which are not known to produce tetracyclines. Out of these four groups, *S. rochei* and *S. exfoliatus* could be considered the main reservoirs of the *otr*(A) and *otr*(B) genes in the screened habitats. Isolates that hybridized with the *otr* probes were obtained not only from polluted habitats, but also from environments with limited or no obvious antibiotic selective pressure such as untreated bulk and rhizosphere soils. Our data showed that *otr*(B) gene was more frequently detected in the samples than *otr*(A) gene. This could imply that the efflux mechanism for resistance to tetracycline in streptomycete populations is dominant over the ribosomal protection mechanism. An interesting property of ribosomal protection is that it does not normally confer high-level tetracycline resistance, as compared to the efflux genes, when cloned into *E. coli*. Thus, the ribosomal protection mechanism of resistance might not provide much survival value in the presence of tetracycline in nature for the enteric bacteria [19]. However, there is no information available referring to levels of resistance that ribosomal protection mechanisms confer to *Streptomyces* sp. to support this idea.

Interestingly, most *otr* amplicons from the isolates shared 100% sequence identity with the sequences of *otr* genes found in *S. rimosus*. The occurrence of identical tetracycline resistance genes in different streptomycete hosts provides additional support for the idea that horizontal gene transfers are the main events that help active exchange of such genes within the *Streptomyces* cluster [18].

In this study, we provided more information on the occurrence of tetracycline resistance genes *otr*(A) and *otr*(B) in a collection of streptomycete isolates from different environmental samples. Although previous studies [22] have addressed the prevalence of specific antibiotic resistances in the environment, none of the studies on tetracycline resistance included streptomycete populations in their investigations. The observation that tetracycline resistance was present in the apparent absence of antibiotic selective pressure (bulk and rhizosphere soil, manure and seawater samples that have no obvious tetracycline contamination but carry streptomycetes with *otr*(A) and *otr*(B) genes) raises the question of how resistance persists. A recent study suggested that bacteria may have been able to adapt to the load of carrying resistance with little or no cost to their fitness [17]. In this scenario, the antibiotic-resistant microbiota would successfully compete with the sensitive phenotypes even in the absence of selection, which would make control of antibiotic resistance even more difficult.

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