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Prevalence of Tetracycline Resistance Genes in Greek Seawater Habitats

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The presence of selected tetracycline resistance (Tc^R) genes was studied in different Greek seawater habitats, originated from wastewater treatment facilities, fishfarm, and coastal environments. The methods employed included assessment of the presence of twelve gene clusters by PCR, followed by hybridization with specific probes, in habitat extracted DNA, Tc^R bacteria, and exogenous isolated plasmids conferring Tc^R. The direct DNA-based analysis showed that *tet*(A) and *tet*(K) genes were detected in all habitats, whilst *tet*(C) and *tet*(E) were present in fishfarm and wastewater effluent samples and *tet*(M) was detected in fishfarm and coastal samples. Resistance genes *tet*(A), *tet*(C), *tet*(K), and *tet*(M) were detected in 60 of the 89 isolates screened. These isolates were identified by fatty acid methyl ester analysis (FAME) as *Stenotrophomonas*, *Acinetobacter*, *Pseudomonas*, *Bacillus*, and *Staphylococcus* strains. The presence of the Tc^R genes in 15% of the bacterial isolates coincided with the presence of IncP plasmids. A habitat-specific dissemination of IncP alpha plasmids in wastewater effluent isolates and of IncP beta plasmids in fishfarm isolates was observed. Exogenous isolation demonstrated the presence of plasmids harbouring Tc^R genes in all the habitats tested. Plasmids were shown to carry *tet*(A), *tet*(C), *tet*(E), and *tet*(K) genes. It is concluded that Tc^R genes are widespread in the seawater habitats studied and often occur on broad host range plasmids that seem to be well disseminated in the bacterial communities.

***Keywords*:** tetracycline resistance genes, seawater samples, exogenous plasmid isolation, polymerase chain reaction analysis, total community DNA

Tetracyclines are broad-spectrum antibiotics and have been used extensively the last 60 years in human and animal therapy (Roberts, 1996; Chopra and Roberts, 2001). Their wide and multipurpose use had enhanced the rapid exchange of resistance genes between members of mixed bacterial populations. The fact that tetracycline resistance (Tc^R) genes are often associated to mobile genetic elements, such as conjugative plasmids and transposons, facilitated their rapid spread across species and genus borders (Schmidt *et al.,* 2001; Roberts, 2005).

Although studies on Tc^R had been primarily focused on clinical bacteria, research interest moved on bacteria from agricultural, animal husbandries, and aquaculture sites, since the dissemination of resistant phenotypes has raised concerns on the effectiveness of tetracycline therapy in such habitats (Huys *et al.*, 2000; Chee-Sanford *et al.*, 2001; Miranda *et al.*, 2003). In particularly, marine environments had attracted much of attention from several scientific groups, since this complex ecosystem act as reservoir by collecting antimicrobial agents and resistant bacteria from a wide range of habitats (Aoki, 1992; Chee-Sanford *et al.*, 2001). Recent studies have focused on the occurrence of tetracycline resistant bacteria in marine habitats and their relationship to population composition of the microbial community (Kim *et al.*, 2004; Furushita *et al.*, 2005; Le *et al.*, 2005). However, more data need to be provided to evaluate the fate of Tc^R genes that are released into such environments.

The aim of this work was to investigate the prevalence of Tc^R genes, of 12 classes, in bacterial communities of Greek marine habitats. Cultivation was employed and combined to culture-independent molecular techniques for monitoring the Tc^R genes in the selected seawater samples. Plasmid analysis was also performed in order to investigate whether the respective genes were associated to broad-host range plasmid groups. This study is to the best of our knowledge the first attempt for the evaluation of Tc^R abundance in natural seawater bacterial communities of Greek marine sites.

Materials and Methods

Bacterial strains and plasmids

The reference plasmids carrying Tc^R genes that were used for testing the primer systems and for generation of probes are listed in Table 1. The following rifampicin and kanamycin resistant mutants were used as recipients in exogenous matings: *Pseudomonas putida* UWC1 (*Gammaproteobacteria*), *Ralstonia eutropha* JMP228 (*Betaproteobacteria*), *Agrobacterium tumefaciens* UBAPF2 (*Alphaproteobacteria*) and *Escherichia coli* CV601 (*Gammaproteobacteria*) (Smalla *et al.*, 2000a). All recipients were *gfp* marked by introducing the mini-transposon vector pAG508 GBF as described by Suarez *et al.* (1997), which was kindly provided by K. Smalla (BBA, Braunchweig, Germany).

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Table 1. Reference plasmids used in the study and primer sets designed for the amplification of *tet* genes

Plasmid (Ref.)	Tc^R gene - Resistance mechanism	Primer sequence (5'-3')	Amplicon size (bp)	T_a (°C)
RP1 (Waters *et al*., 1983)	*tet*(A)-Efflux	F: GGCCTCAATTTCCTGACG R: AAGCAGGATGTAGCCTGTGC	372	58
pRT11 (2,7-kb *Hpa*I fragment from lambda::Tn*10* cloned into pVH51) (Marshall *et al*., 1987)	*tet*(B)-Efflux	F: GAGACGCAATCGAATTCGG R: TTTAGTGGCTATTCTTCCTGCC	228	58
pBR322 (Bolivar *et al*., 1977)	*tet*(C)-Efflux	F: TCCTTGCATGCACCATTCCC R: AACCCGTTCCATGTGCTCG	379	59
pSL106 (3,05-kb HindIII-PstI fragment from RA1 cloned into pACYC177) (Marshall *et al*., 1987)	*tet*(D)-Efflux	F: GGATATCTCACCGCATCTGC R: CATCCATCCGGAAGTGATAGC	436	58
pSL1504 (pSL1456 cloned into pACYC177) (Marshall *et al*., 1987)	*tet*(E)-Efflux	F: TCCATACGCGAGATGATCTCC R: CGATTACAGCTGTCAGGTGGG	442	58
9-kb *Hin*dIII fragment from pJA8122 of *Vibrio anguillarum* into pUC119 (Zhao and Aoki, 1992)	*tet*(G)-Efflux	F: TTTCGGATTCTTACGGTC R: TCCTGCGATAGAGCTTAGA	858	50
pVM111 (Hansen *et al*., 1993)	*tet*(H)-Efflux	F: GTGATGTGACTCCCGCTAA R: CCATACCTCCTGCCGCTAA	569	55
pAT102 (Hansen *et al*., 1993)	*tet*(K)-Efflux	F: TTATGGTGGTTGTAGCTAGAAA R: AAAGGGTTAGAAACTCTTGAAA	347	52
pUC18 (Widdowson *et al*., 1996)	*tet*(L)-Efflux	F: GT(AC)GTTGCGCGCTATATTCC R: GTGAA(AC)G(AG)(AT)AGCCCACCTAA	696	55
pUC8 (Widdowson *et al*., 1996)	*tet*(M)-Ribosomal protection	F: GT(AG)A(CT)GAACTTTACCGAATC R: ATCG(CT)AGAAGCGG(AG)TCACT	615	50
pUC19 (Widdowson *et al*., 1996)	*tet*(O)-Ribosomal protection	F: AATGAAGATTCCGACAATTT R: CTCATGCGTTGTAGTATTCCA	780	48
pGEM carrying *tet*(O) gene from *Butyrivibrio fibrisolvens* (Barbosa *et al*., 1999)	*tet*(T)-Ribosomal protection	F: GTATTTCATGGTTCGGCTTTA R: AATCCCGTCATATTTCTTACC	341	50

Sampling and sample processing

Seawater samples were collected from four Greek marine habitats, twice a year from 1999 until 2001 and sampling was repeated the years 2003 and 2005. The selected sites were: a) a fishfarm at Epidavros, b) the marine site where the outflow of the wastewater treatment plant in Psyttaleia is situated (Saronic Bay NW of Athens), c) a coastal site at Eretria (Evia Island) a tourist destination mainly in summer, without a wastewater treatment plant and d) a pristine coastal site at Fleves Island (Saronic Bay S of Athens) which is uninhabited. All sampling sites and sampling procedure have been previously described in detail (Heuer *et al.*, 2002; Van Overbeek *et al*., 2002).

Isolation of Tc^R colonies

Heterotrophic bacteria in seawater samples were recovered using the spread plate method. Serial 10-fold dilutions were prepared from the bacterial suspensions and aliquots (0.1 ml) were plated in duplicate onto Marine Agar (Difco, UK) amended with 10 μg/ml of tetracycline (Sigma, Germany). Plates were incubated for 3 to 7 days at 20°C. Distinct colonies with different morphology were randomly selected and isolated in pure culture on Marine Agar. Purified strains were then stocked in nutrient broth with 15% glycerol at -20°C.

Identification of Tc^R bacterial isolates

For each seawater sample morphologically different Tc^R colonies were randomly selected and streaked on Trypticase Soy Broth (Becton Dickinson, USA) supplemented with Bacto-Agar (15 g/L, Difco, UK). In total 89 Tc^R strains were identified or classified by fatty acid methyl ester analysis (FAME) analysis using the Microbial Identification System (MIS, MIDI Inc., USA). Out of these isolates, 36 originated from fishfarm, 23 from wastewater effluent, 14 from Eretria and 16 from Fleves Island samples.

Susceptibility testing

Antibiotic susceptibility was performed using the Kirby-Bauer disk diffusion method described by the Clinical and Laboratory Standard Institute (CLSI) (2006a, b). Mueller-Hinton Agar (Difco, UK) was utilised as test medium and twelve antimicrobial agents were selected as representatives of different antibiotic classes: ampicillin (Amp) 10 μg; ka-

Table 2. Distribution of Tc^R genes in total community DNA of seawater samples

Sample origin	Tc^R genes[a]											
	tet(A)	*tet*(B)	*tet*(C)	*tet*(D)	*tet*(E)	*tet*(G)	*tet*(H)	*tet*(K)	*tet*(L)	*tet*(M)	*tet*(O)	*tet*(T)
Fishfarm	+	-	+	-	+	-	-	+	-	-	-	-
Wastewater effluent	+	-	+	-	+	-	-	+	-	+	-	-
Eretria	+	-	-	-	-	-	-	+	-	+	-	-
Fleves Island	+	-	-	-	-	-	-	+	-	-	-	-

[a] +, presence; -, absence

namycin (K) 30 μg; neomycin (N) 30 μg; streptomycin (S) 25 μg; gentamicin (Gm) 30 μg; nalidixic acid (Na) 30 μg; vancomycin (V) 30 μg; erythromycin (E) 15 μg; minocycline (Mh) 30 μg; novobiocin (Nv) 30 μg; rifampicin (Rd) 30 μg and fusidic acid (Fd) 10 μg. Aminoglycosides were represented by five agents (K, N, S, Gm, and Nv), since all of them have been extensively used in agricultural practice in Greece. For other antimicrobial groups, such as cephalosporins, it has been decided not to be included in this study as their use is mainly clinical. All were products of Sigma. Minimal inhibitory concentration (MIC) value for Tc was also estimated for each isolate by E-tests (Biomerieux, France) according to the guidelines of the CLSI (2006a, b). In the absence of standard interpretative schemes for environmental isolates, the susceptibility of the seawater isolates was interpreted comparing MIC values to the breakpoint concentrations suggested for the standard strains *E. coli* ATCC 25922 or *Staphylococcus aureus* ATCC 29213 (CLSI, 2006a, b).

Exogenous isolation of Tc^R plasmids

Exogenous plasmid isolations were performed shortly after sampling using the recovered bacterial fraction, as described by Hill *et al.* (1996) and Smalla *et al.* (2000a). To select for Tc^R transconjugants serial dilutions were plated on Plate Count Agar (PCA, Merck, Germany) supplemented with cycloheximide (200 mg/L) and nystatin (50 mg/L), to prevent fungal growth, with the addition of K (50 mg/L), Rd (50 mg/L) and Tc (10 mg/L) to select for putative transconjugants.

DNA extraction procedures

Total community DNA was extracted from the bacterial fraction which was concentrated on membrane filters and stored at -20°C immediately after filtration, following a modified protocol of Fuhrman and co-workers (1993) as previously described (Nikolakopoulou *et al.*, 2005). Total genomic and plasmid DNA were extracted from reference strains, Tc^R isolates and putative transconjugants according to the genomic DNA extraction procedure (Ausubel *et al.*, 1989) and the modified Ish-Horowitz plasmid extraction protocol (Götz *et al.*, 1996).

PCR-based detection of Tc^R genes

Twelve PCR primer sets were used to amplify selected genes coding for tetracycline efflux pumps or ribosomal protection proteins (Table 1). PCR assays amplified the respective genes from either total community DNA, genomic or plasmid DNA and were performed as previously described (Nikolakopoulou *et al.*, 2005). Amplified fragments were resolved by agarose gel (1%) electrophoresis in TBE buffer followed by ethidium bromide staining (Sambrook *et al.*, 1989). PCR-amplified fragments were Southern blotted and hybridized with digoxigenin-labelled probes. Probes were generated by random prime labelling of PCR products generated from reference strains, listed in Table 1 (ca. 300 μg) with digoxigenin dUTP according to the instructions of the manufacturer (Roche, Germany) (Nikolakopoulou *et al.*, 2005).

Plasmid replicon-specific PCR

Plasmids from Tc^R isolates as well as from putative transconjugants were tested with plasmid IncP, IncQ, IncN, and IncW group-specific PCR systems as described by Götz *et al.* (1996). Plasmid DNA extracts were, thus, subjected to four different PCR reactions amplifying: the backbone genes *trf*A1 and *ori*T (both IncP), genes *rep* and *ori*T (both IncQ), genes *ori*T and *ori*V (both for IncW) and *rep*B for the IncN group according to Götz *et al.* (1996). PCR products were analysed, Southern blotted and hybridized with the respective probes as described above. These digoxigenin-labelled probes were generated by random prime labelling of PCR products from the following reference plasmids: P4 (IncP alpha), R751 (IncP_beta), RN3 (IncN), and R388 (IncW).

Results

Detection of Tc^R genes in total community DNA

High-quality total community DNA was obtained from the four marine habitats as shown by the appearance of high molecular mass bands (>20 kb) in agarose gels (data not shown). PCR amplification of these DNA samples followed by hybridization with the relevant probes showed positive signals with multiple, sometimes up to five, out of twelve, PCR systems (Table 2). This result indicated the presence of multiple Tc^R genes in most of the habitats investigated. *tet*(A) and *tet*(K) genes were present in all habitats. Screening of the other Tc^R genes showed that *tet*(C) and *tet*(E) were abundant in fishfarm and wastewater effluent samples, whilst *tet*(M) was related to wastewater effluent and Eretria environment.

Analysis of Tc^R colonies in the habitats studied

In total, 89 resistant colonies were picked as representatives and were analyzed for the presence of Tc^R genes by PCR and DNA-DNA hybridization using the respective probes.

Table 3. Representative bacterial isolates obtained from different samples that probed positive with the selected Tc^R genes

Tc^R genes	Origin of sample	Strain code	Tc^R isolates (MIS[a])	MIC (μg/ml) for Tc[b]	Plasmid detected[c]	Inc group detected
tet(A)	Fishfarm	FF9	*Stenotrophomonas maltophilia* (0.403)	>256	+	-
	Fishfarm	FF22	*S. maltophilia* (0.651)	32	+	IncP beta
	Eretria	ER40	*S. maltophilia* (0.321)	24	-	-
tet(C)	Fishfarm	FF18	*Leclercia adecarboxylata* (0.932)	96	+	IncP beta
	Fishfarm	FF24	*S. maltophilia* (0.427)	6	+	IncP beta
	Fishfarm	FF33	*S. maltophilia* (0.966)	12	+	IncP beta
tet(K)	Wast. effluent	WW78	*Acinetobacter lwoffii* (0.902)	ND[d]	-	-
	Wast. effluent	WW80	*Acinetobacter genospecies* (0.922)	ND	-	-
	Wast. effluent	WW86	*Bacillus cereus* (0.471)	96	+	-
	Fishfarm	FF7	*S. maltophilia* (0.498)	192	+	IncP beta
	Fishfarm	FF19	*Bacillus lentimorbus* (0.895)	12	-	-
	Fishfarm	FF27	*S. maltophilia* (0.909)	24	-	-
	Fishfarm	FF35	*S. maltophilia* (0.953)	12	+	-
	Eretria	ER38	*Staphylococcus epidermidis* (0.713)	16	+	-
	Eretria	ER45	*Staphylococcus warneri* (0.860)	32	+	-
	Fleves Island	FL51	*S. maltophilia* (0.878)	24	+	-
	Fleves Island	FL56	*Bacillus megaterium* (0.925)	24	+	-
	Fleves Island	FL60	*S. maltophilia* (0.860)	ND	-	-
tet(A) and	Wast. effluent	WW69	*S. maltophilia* (0.959)	>256	-	-
tet(K)	Fishfarm	FF29	*Pseudomonas aeruginosa* (0.814)	48	+	-
	Fleves Island	FL57	*St. epidermidis* (0.911)	24	+	-
tet(K) and	Wast. effluent	WW88	*Bacillus megaterium* (0.851)	16	+	-
tet(M)	Eretria	ER37	*Staphylococcus hominis* (0.871)	16	+	-
	Eretria	ER43	*St. hominis* (0.914)	24	-	-
	Eretria	ER46	*St. hominis* (0.863)	64	+	-
	Eretria	ER49	*St.warneri* (0.804)	6	-	-
None of the *tet*	Wast. effluent	WW68	*S. maltophilia* (0.498)	ND	-	-
genes found	Wast. effluent	WW75	*Ochrobactrum anthropi* (0.844)	32	-	-
	Wast. effluent	WW79	*Micrococcus luteus* (0.898)	>256	-	-
	Fishfarm	FF10	*S. maltophilia* (0.978)	16	+	-
	Fishfarm	FF2	*Microbacterium lacticum* (0.929)	8	-	-
	Eretria	ER50	*S. maltophilia* (0.361)	48	+	-
	Fleves Island	FL55	*S. maltophilia* (0.881)	192	-	-
	Fleves Island	FL59	*Rhodococcus fascians* (0.860)	16	-	-
	Fleves Island	FL61	*Xanthomonas hortorum* (0.872)	6	-	-

[a] MIS, similarity of the fatty acid methyl ester profile to TSBA database entry of the Microbial Identification System (MIDI Inc.), highest similarity in case of several isolates of a species.
[b] Isolates with Tc MIC<6 μg/ml were considered sensitive; if it was 6 to 12 μg/ml were considered intermidiate and if it was £16 were resistant according to CLSI (2006a, b).
[c] Plasmid band in agarose gel
[d] ND, Not determined

Sixty of the 89 isolates showed signals with one or more of the *tet* probes tested. It was revealed that *tet*(K) was the dominant gene, present as a single *tet* gene in 29 isolates (10 from fishfarm, 9 from wastewater, 4 from Eretria, and 6 from Fleves Island), but was also detected together with *tet*(A) in 1 isolate from fishfarm, 1 from wastewater, and 2 from Fleves Island. *tet*(A) was present as a single *tet* gene in 9 isolates, 3 from fishfarm, 3 from wastewater, 2 from Eretria, and 1 from Fleves Island. As a single gene, *tet*(M) was detected in 2 isolates originating from the wastewater effluent site, whereas together with *tet*(K) was present in 3 wastewater strains. Incidence of the *tet*(C) gene was only detected in 4 fishfarm isolates (Table 3).

Plasmid extraction from the 60 isolates, carrying a specific *tet* gene, followed by agarose gel electrophoresis, revealed clear plasmid bands in 37 isolates with sizes >40 kb (data not shown). Determinants for IncP-type plasmids were observed in 10 isolates and the presence of these determinants in all cases coincided with *tet* genes. Among them, IncP beta-type plasmids were present in 7 fishfarm isolates, whilst IncP alpha-type plasmids were observed in 3 wastewater effluent isolates (Table 3).

Identification of the isolates revealed that they belong to phylogenetically distinct species. Among the 89 Tc^R isolates screened, 64% were Gram-negative (57 isolates) and isolates related to *S. maltophilia* species were abundantly observed in all samples. Referring to fishfarm isolates, 67% of them were characterized as *S. maltophilia* strains (24 isolates),

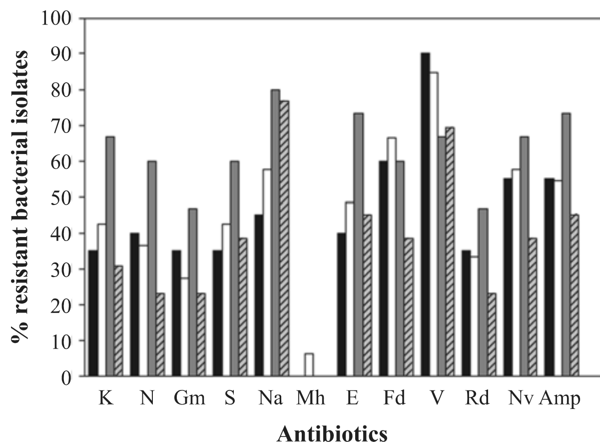

Fig. 1. Antibiotic resistance patterns of Tc^R seawater bacterial isolates in twelve different antibiotics [Fishfarm n=36 (■), Wastewater effluent n=23 (□), Eretria n=14 (▩), Fleves Island n=16 (▨)].

whereas this quotient was 43% for the Eretria isolates (6 isolates), 38% for Fleves Island (6 isolates), and 13% for the isolates originated from the wastewater effluent environment (3 isolates). Other bacterial groups such as *Acinetobacter*, *Pseudomonas*, *Leclercia*, *Ochrobactrum*, *Bacillus*, and *Staphylococcus* with few representatives were also present (Table 3).

Antimicrobial resistance

Tc^R bacterial isolates were further assayed for antibiotic resistance. Among the 89 tested strains (32 Gram-positive and 57 Gram-negative), 96% were resistant to one or more of the twelve selected antibiotics, whereas only 3 fishfarm isolates (two *S. maltophilia* and one *Bacillus lentimorbus*) appeared to be totally susceptible to all twelve antibiotics studied.

Comparison of samples from the four selected marine habitats showed that Tc^R bacteria isolated from the fishfarm seawater samples showed resistance to the ten of the twelve antibiotics tested, except to Na and Mh. As for the wastewater effluent site more than 50% strains were resistant to V (84.6%), Fd (66.6%), Na (57.6%), Nv (57.6%), and Amp (54.5%). Referring to Eretria isolates more than 50% of strains were resistant to nine out of the twelve tested antibiotics: K, N, S, Na, E, Fd, V, Nv, and Amp. Comparison of samples from the highly disturbed (wastewater effluent and fishfarm) and the less polluted sites (Fleves Island and Eretria) did not show higher percentages of bacterial resistance to the studied antibiotics (Fig. 1).

MIC values of tetracycline were determined for all 89 Tc^R isolates and they ranged from 6 μg/ml to >256 μg/ml (Table 3). Our results did not reveal any relation between the MIC value and the bacterial taxon of the isolate or the presence of specific *tet* gene (Table 3).

A total of 37 different multiple-drug resistance patterns were identified (data not shown). 25% of wastewater effluent strains were multi resistant to three, four, five, and six antibiotics, while 35% of strains of the same origin were resistant to ten and eleven out of twelve antibiotics tested. Referring to the fishfarm strains 78.8% were multi resistant to three or more antibiotics, while 30% showed resistance to ten and eleven agents. Similarly, 66.6% of strains originated from Fleves Island and 38.4% of Eretria strains were multi resistant to five or more antibiotics.

Exogenous Tc^R plasmid isolation

Bacterial fraction originated from all samples was used in exogenous plasmid isolation experiments. Recipient strains acquired mobile Tc^R genes, from all habitats, at transfer frequencies that ranged from 15.3×10^{-5} to 1.7×10^{-7}. Putative transconjugants could be discriminated from indigenous tetracycline, rifampicin, and kanamycin resistant bacteria by their fluorescence. Tc^R plasmids, sized between 40~80 kb, were observed in 80 transconjugants (24 *P. putida* UWC1, 23 *E. coli* CV601, 11 *A. tumefaciens* UBAPF2, and 22 *R. europha* JMP228 recipients).

Characterization of the Tc^R genes present in plasmids, obtained by exogenous isolation, indicated that 59 plasmids possessed one or more of the Tc^R genes tested. *tet*(A) was the dominant gene found as a single *tet* gene (on 36 plasmids) or together with *tet*(K) gene (on 7 plasmids). *tet*(A) carrying plasmids originated from all sampling sites. *tet*(K) was also detected alone on 11 plasmids from all sites (2 from fishfarm, 3 from wastewater, 4 from Eretria, and 2 from Fleves Island). *tet*(C) was only present on 3 plasmids isolated from the fishfarm environment.

27 plasmids obtained exogenously (33.7%) showed signals in PCR reactions with primers directed against plasmid 'backbone' genes, specific for the IncP group. 20 plasmids gave positive hybridization signals with either one or both IncPalpha-specific probes. These plasmids were isolated from wastewater, Fleves Island and Eretria samples, and were screened to carry *tet*(A) and *tet*(K) genes. Seven exogenously isolated plasmids from the fishfarm site gave positive hybridization results with IncPbeta-specific probes and were shown to carry the same *tet* genes, as well.

Discussion

The apparent increase of the occurrence of antibiotic resistance among bacteria from various environments during the past years and its possible implication in public health has led to an intensified surveillance of bacterial resistance in many countries (Aoki, 1992; Chelossi *et al.*, 2003). In the field of marine habitats and aquaculture, both therapeutic and environmental problems have been addressed, as antimicrobial agents are released into the surrounding water (Smith *et al.*, 2004).

Screening for the twelve selected Tc^R genes (Table 1) in total community DNA from the seawater samples revealed that several genes were abundant in almost all sampling sites, irrespective to their relation to clinical environment or to the degree of human activity. The *tet*(A) and *tet*(K) genes were the most widespread genes in the habitats studied. These two genes have been reported in clinical, veterinary, as well as aquatic habitats and they are related to mobile genetic elements in the isolates screened (Chopra and Roberts, 2001; Miranda *et al.*, 2003). Three other genes, *tet*(C), *tet*(E), and *tet*(M) were also present in half of the

habitats investigated. These genes have also been described in clinical isolates by Trzcinski and co-workers (2000), whilst reports on their occurrence in the marine environment have been published recently (Chee-Sanford *et al.*, 2001; Kim *et al.*, 2004).

Tc^R bacteria were found in all studied samples and isolates were grouped by FAME analysis within a range of 12 bacterial species. However, the taxonomic composition of population (from both Gram-negative and Gram-positive groups) varied among the four sites. Analysis revealed that *S. maltophilia* was the dominant species in the fishfarm samples, as it represented the 67% of the characterized isolates. It was, also, frequently present in the samples of Eretria (42%) and Fleves Island (36%), data that are in accordance to several reports on marine habitats (Goni-Urizza *et al.*, 2000; Huys *et al.*, 2000; Schmidt *et al.*, 2001; Furushita *et al.*, 2005; Piccini *et al.*, 2006). Gram-positive isolates belonged to *Staphylococcus* and *Bacillus* genera.

Results showed that a permanent supply of antibiotic-resistant bacteria to the environment is established and might lead to a continuous dissemination and accumulation of resistant organisms in environmental water (Da Silva *et al.*, 2006). Four different Tc^R determinants *tet*(A), *tet*(C), *tet*(K), and *tet*(M) occurred among the Tc^R isolates and in a few cases more than one determinant was detected in a single strain. The prevalence of the *tet*(K) gene has not been reported up to date in any environmental study concerning the above mentioned species. Previous reports on Tc^R demonstrated that *tet*(A), *tet*(E), and *tet*(M) genes were usually dominant in disturbed seawater samples (Chee-Sanford *et al.*, 2001; Miranda *et al.*, 2003). This is the first report of the presence of the *tet*(K) gene in *Stenotrophomonas*, *Pseudomonas*, and *Acinetobacter* isolates. The finding that a variety of species, including different bacterial taxa, carry *tet* genes indicated that Tc^R is probably widely spread among different prokaryotic groups. Screening of total community DNA and Tc^R isolates had not led to the same results, referring to the detection of specific *tet* genes in both analyses. This is expected, since due to the unculturability of the majority of marine bacteria, only a small bacterial fraction of the whole community was represented in the isolates that were further analysed (Fuhrman *et al*., 1993).

The presence of *tet* genes often coincided with the presence of plasmids in the resistant isolates analyzed. Isolation of Tc^R plasmids and further characterization showed that they belonged to IncP group. IncP plasmids are commonly observed in natural environments and have been isolated from marine habitats (Dahlberg *et al.*, 1997; Sobecky, 1999; Smalla *et al.*, 2000b). Additionally, the identification of plasmids which could not be allocated to known Inc groups is not unexpected. Several recent studies have indicated that most of the plasmids found in bacterial communities of terrestrial environments, as well as in aquatic habitats such as the river epilithon and the marine environment, belong to so far undescribed groups (Hill *et al.*, 1992; Hill *et al.*, 1996; Dahlberg *et al.*, 1997; Smit *et al.*, 1998; Smalla *et al.*, 2000b).

Exogenous plasmid isolation revealed that *tet*(A) dominated this horizontal gene pool. This gene, which was also abundantly found in total community DNA, is probably the most commonly associated with mobile elements. As the *tet*(A) family is one of the best described and present in many different habitats, our data add information on the dissemination of these genes in natural ecosystems (Rhodes *et al.*, 2000; Chopra and Roberts, 2001). The vast majority of the plasmids obtained exogenously possessed at least one Tc^R gene, whilst some of them gave no positive signal with the applied detection systems. These plasmids might carry either known genes that were not included in the study, or unknown Tc^R genes. The finding of multiple Tc^R genes in a number of conjugative plasmids correspond to previously reported results on the presence of multiple Tc^R genes on mobile elements (Warsa *et al.*, 1996; Kim *et al.*, 2004).

The increasing problem of bacterial antimicrobial resistance demands a well coordinated effort by all countries to decrease antibiotic use and limit resistance spread. In this respect, regular and continuous antibiotic resistance surveillance should be established in order to monitor the dissemination of resistance genes to natural ecosystems.

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