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Short Communication

New molecular variants of epsilon and beta IncP-1 plasmids are present in estuarine waters

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ABSTRACT

In this work the presence of broad-host-plasmids in an estuary in Portugal has been investigated. *Pseudomonas putida* KT2442 was used as model recipient bacteria in biparental matings with tetracycline and mercury to select for resistance phenotypes. As a result, 7 transconjugants were shown to carry broad-host-plasmids from the IncP-1 group, as seen by PCR amplification of the *trfA* gene. Sequence analysis confirmed the isolation of 4 plasmids from β-1 subgroup and 3 assigned to the recently described ε subgroup. To our knowledge this is the first report concerning the detection and isolation of IncP-1β and ε plasmids in estuarine waters. Moreover it is shown that, even though the retrieved plasmids are phylogenetically close to previously characterized plasmids, such as pB10 and pKJK5, respectively, they constitute new molecular variants.

1. Introduction

Broad host range (BHR) plasmids are known for being main facilitators of gene transfer across genera, phylum and domains (Sorensen et al., 2005). Their abundance has been related with spread and maintenance of adaptive traits in microbial communities supported by selective pressure forces at play (Smalla et al., 2006; Moura et al., 2010; Schmidt et al., 2001). A set of primers is available for the PCR amplification of replicon specific DNA regions from BHR plasmids belonging to incompatibility groups N, Q, W, and P (Gotz et al., 1996) and more recently for IncP plasmids subgroups, IncP-1β, α, ε, δ, and γ (Bahl et al., 2009). IncP plasmids (IncP-1 in *Pseudomonas* classification system) have attracted attention due to their diversity in accessory genes, ranging from antibiotic and mercury resistance to degradation of xenobiotic compounds, and to their wide host range, being able to transfer, usually from gram-negative bacteria, to gram-positive bacteria, cyanobacteria and even yeasts (Smalla and Sobecky, 2002; Trieu-Cuot et al., 1987; Kreps et al., 1990; Heinemann and Sprague, 1989).

Initially BHR plasmids have been detected in culturable bacteria but since only a small fraction of bacteria are amenable to be cultured (Amann et al., 1995), culture independent plasmid capture methods were developed to circumvent this bias (Bale et al., 1988). Exogenous plasmid isolations, have been used in a set of environments frequently yielding BHR plasmids (Top et al., 1994; Droge et al., 2000; Smalla and Sobecky, 2002; Smalla et al., 2006; Bahl et al., 2007).

More information is needed on evolutionary relationships, phenotypes and ecological roles of BHR plasmids from different habitats to understand how they shape microbial communities and ecosystem. Also, their molecular diversity may be underestimated since the current available data refers to plasmids isolated from clinical environments and few other environments (Frost et al., 2005). Estuaries are highly productive and dynamic environments

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and can be of interest for resistance plasmid investigations (Ducklow and Carlson, 1992) since they are considered reservoirs of antibiotic resistance (Henriques et al., 2006; Laroche et al., 2007). Up until now data on plasmid mediated horizontal gene transfer is limited and nonexistent is the association with BHR plasmids. Previous investigations (Dahlberg et al., 1997; Dahlberg et al., 1998; Heuer et al., 2002) showed that gene transfer is a part of natural processes that occur between bacteria in marine environments. As a result, a collection of marine conjugative plasmids was obtained from which nucleotide sequencing allowed the identification of 2 IncP-1 plasmids from marine biofilm (Dahlberg et al., 1997) representing a new subgroup (ξ) (Norberg et al., 2011).

Overall, the few investigations performed so far about conjugative gene transfer in freshwater and marine waters suggest higher frequencies in surface microlayers (SML) than in underlying waters (UW) (Jones et al., 1991; Dahlberg et al., 1997). SML concentrates organic matter and films that favor microbial growth in extreme conditions, such as under high UV radiation and higher concentration of pollutants (Cunliffe et al., 2010). As a result, SML bacterial communities might carry different horizontal gene pool than bacterioplankton as a result of the different selective pressures forces present, as suggested previously by Hermansson and co-workers (1987) in one of the first plasmid reports from marine SML. It seems plausible to suggest that BHR plasmids can be more easily detected and isolated at the air–water interface than in bulk water. So, the aim of the present work is to investigate the occurrence of BHR plasmid in estuarine waters by sampling in SML and UW using culture independent methods.

2. Materials and methods

2.1. Exogenous isolation of conjugative plasmids

Estuarine waters at Ria de Aveiro, Portugal, (40°38′N, 8″45′W) were used to sample for conjugative plasmids. Samples were taken from the shoreline of two sampling sites, *Cais do Sporting* (CS) and *Cais do Chegado* (CC), during Spring and Summer of 2009 during day, at low tide, with wind below 3–4 m/s and in dry periods. Salinity and temperature was determined in situ using a WTW Conductivity Meter model LF 196 (WTW, Weilheim, Germany) and measured using the Practical Salinity Scale.

To collect the SML (60–100 μm) both acrylic and glass plates (Agogué et al., 2004; Santos et al., 2009) were used. UW samples were collected in 2 L dark autoclaved bottles, from the depth of about 40 cm. To prevent from SML sample contamination, the bottle cap was opened and closed with the bottle submerged. Samples were kept cold and in the dark during transport and processed within 3 h after sampling.

Bacterial strains as well as plasmids used throughout this study are listed in Table 1. Rifampicin (100 μg/ml) and kanamycin (50 μg/ml) resistant *Pseudomonas putida* KT2442 was used as model recipient bacteria in biparental matings. Plasmid donor bacteria were from neuston (group of organisms inhabiting the SML) and plankton samples. Conditions for mating were without added nutrients and to starved recipient cells according to Dahlberg et al. (1997) except otherwise noted. When possible, SML and UW sample volumes were adjusted to give the same number of donor cells, avoiding a bias of initial cell densities in the comparison of transconjugants ratios. Recipient cells were 4×10^9 in all experiments. Determination of total number of cells was by acridine orange direct counts (AODC) (Francisco et al., 1973). Also, when necessary and in order to remove large particles both samples were pre-filtered through sterilized filter paper. After filter matings (48 h, 20 °C) cells were washed and resuspended in 5 mL NSS and serial diluted. Transconjugants were isolated on TSA with mercury chloride (25 μg/mL) or tetracycline (30 μg/mL) both supplemented with rifampicin and kanamycin and controls were according to Dahlberg et al. (1997). The plates were incubated at 25–28 °C for 48 h. Putative transconjugants were verified by analyzing repetitive extragenic palindromic-PCR (REP-PCR) fingerprints, obtained as described before (Henriques et al., 2006).

2.2. Identification of broad-host-range specific sequences

PCR detection of replicon specific sequences of IncN, Q and W plasmids was according to Gotz et al. (1996), except that 0.5 U Taq polymerase (Fermentas) per reaction was used. Primers systems are presented in Table 2. PCR products were dot blotted onto a Hybond-N membrane (Amersham Biosciences) using standard protocols (Sambrook et al., 1989). Hybridization of PCR products was carried out using PCR derived digoxigenin-labeled probes using as template positive controls for each incompatibility group (Table 1) and was performed according to the

Table 1
Bacterial strains and plasmids used in this study.

Strains	Plasmid	Comments	Source
Pseudomonas putida KT2442	–	Recipient strain	Kornelia Smalla-Julius Kuhn Institut, Germany
Escherichia coli Top10	–	Electrocompetent	Invitrogen
Escherichia coli K12	pULB2432	IncN plasmid group	Elisabeth Grohmann-Technische Universität Berlin, Germany
Escherichia coli DH5α	R388	IncW plasmid group	Elisabeth Grohmann-Technische Universität Berlin, Germany
Escherichia coli JE723	pJE723	IncQ plasmid group	Elisabeth Grohmann-Technische Universität Berlin, Germany
Escherichia coli JM109	pB10	IncP-1β plasmid group	Elisabeth Grohmann-Technische Universität Berlin, Germany
Escherichia coli SCS1	RP4	IncP-1α plasmid group	Elisabeth Grohmann-Technische Universität Berlin, Germany
Escherichia coli CSH26	pKJK5	IncP-1ε plasmid group	Soren J. Sorensen-University of Copenhagen, Denmark
Escherichia coli DHIOB	pQKH54	IncP-1γ plasmid group	Soren J. Sorensen-University of Copenhagen, Denmark
Achromobacter xylosoxidans subsp.	pEST4011	IncP-1δ plasmid group	Eve Vedler,-Tartu University, Estonia

Table 2
Primer systems used for the amplification of BHR plasmids.

Primer sequence	Annealing temp (°C)	Inc group	Region	Product size (bp)	Reference
F w: 5′-TTCACSTTCTACGAGMTKTGCCAGGAC Rv: 5′-GWCAGCTTGCGGTACTTCTCCCA-3′	67	IncP1-α IncP1-β IncP1-ε	*trfA*	281	Bahl et al. (2009)
Fw: 5′-TTCACTTTTTACGAGCTTTGCAGCGAC-3′ Rv: 5′-GTCAGCTCGCGGTACTTCTCCCA-3′	67	IncP1-γ	*trfA*-γ	281	Bahl et al. (2009)
Fw: 5′-TTCACGTTCTACGAGCTTTGCACAGAC-3′ Rv: 5′-GACAGCTCGCGGTACTTTTCCCA-3′	67	IncP1-δ	*trfA*-δ	281	Bahl et al. (2009)
Fw: 5′-TCTGCATCATTGTAGCACC-3′ Rv: 5′-CCGTAGTGTTACTGTAGTGG-3′	51	IncW	*oriT*	317	Gotz et al. (1996)
Fw: 5′-AGTTCACCACCTACTCGCTCCG-3′ Rv: 5′-CAAGTTCTTCTGTTGGGATTCCG-3′	55	IncN	*rep*	164	Gotz et al. (1996)
Fw: 5′-CTCCCGTACTAACTGTCACG-3′ Rv: 5′-ATCGACCGAGACAGGCCCTGC-3′	57	IncQ	*oriV*	436	Gotz et al. (1996)
Fw: 5′-TTCGCGCTCGTTGTTCTTCGAGC-3′ Rv: 5′-GCCGTTAGGCCAGTTTCTCG-3′	57	IncQ	*oriT*	191	Gotz et al. (1996)

manufacturer's instructions (Roche Diagnostic, Mannheim, Germany). Detection of replicon specific sequences (Table 2) of IncP-1 plasmids from subgroups α, β, ε, γ and δ was done by PCR (Bahl et al., 2009) using 1 U of Taq polymerase (Promega) per reaction. Positive results were verified by nucleotide sequencing of the amplification products.

Positive (Table 1) and negative (*P. putida* KT2442) controls were included to confirm the specificity of the detections. Detection of the PCR amplification products was performed by electrophoresis on a 1.5% agarose gel in TAE buffer and visualized by ethidium bromide staining.

2.3. Plasmid transfer, isolation and restriction analysis

Plasmids from transconjugants were transferred to *Escherichia coli* Top10 competent cells (Invitrogen, Carlsbad, CA, USA) by electroporation. Transformants were selected by plating with the appropriate selective marker and confirmed by amplification of *trfA* gene fragment.

Plasmid DNA was isolated with the Qiagen Plasmid Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions with modifications. Eight millilitres of starter cultures were used instead of 3 and 0.6 mL of buffers P1, P2 and P3 were added instead of 0.3 mL.

The genetic diversity of the IncP-1 plasmids was compared using NotI (MBI Fermentas, Vilnius, Lithuania) digests separated by agarose gel electrophoresis (1%). Restriction profiles were compared with NotI digests from IncP-1β (pB10) and IncP-1ε (pKJK5) plasmids.

2.4. Determination of antibiotic and mercury salts resistance patterns

The antibiotic resistance patterns of the transformants were determined by the agar disk diffusion method on Mueller–Hinton agar (Merck, Darmstadt, Germany). Antibiotics were chosen according with the antibiotic resistance phenotypes associated with IncP-1β and ε plasmids studied so far. Tests were carried out using disks containing the following antibacterial agents: amoxicillin (25 μg), amoxicillin and clavulanic acid (30 μg), tetracycline (10 μg), chloramphenicol (10 μg), trimethoprim-sulfamethoxazole (25 μg), cephalotin (30 μg) and aztreonam (30 μg) (Oxoid, Basingstoke, UK). Plates were incubated at 37 °C for 24 h and the transformants were classified as sensitive, intermediate or resistant according with the guidelines provided by the Clinical Laboratory Standards Institute (CLSI, 2010).

Transformants harboring plasmids encoding resistance to tetracycline (pMLUA1, pMLUA2, pMLUA3 and pMLUA4) were tested for mercury resistance. Strains were picked with sterile toothpicks and streaked on TSA plates supplemented with 25 μg/mL of $HgCl_2$ and incubated at 37 °C for 24 h.

2.5. Sequencing and phylogenetic analysis of the amplified trfA gene fragment

The PCR products obtained using tfrA primers were purified (JETQUICK PCR purification spin kit- Genomed, Löhne, Germany) and sequenced (StabVida-Oeiras, Portugal). Nucleotide sequence analysis was carried out using BLAST (Altschul et al., 1997) multiple alignments were constructed with the CLUSTALX (Thompson et al., 1997) and parsimony trees based on the distance parameter were constructed using the PAUP4.0b Program (Swofford, 2002). Bootstrap values from 1000 replicates were also obtained. The sequences of the *trfA* gene fragments from plasmids pMLUA1-7 have been submitted to DDBJ/EMBL/GenBank nucleotide sequence database with the accession numbers JF907414, JF907415, JF907416, JF907417, JF907418, JF907419 and JF907420, respectively.

3. Results and discussion

Capture of tetracycline resistance was successful for SML samples from CS and CC sampling sites (two transconjugants isolated in each sample) with transfer efficiencies of 1.3×10^{-7} and 1.8×10^{-8} transconjugants per donor cell, respectively. Capture of mercury resistance was detected for one sampling site (CC) with a transfer frequency of 1.8×10^{-6} (32 transconjugants for SML and 43 for UW samples). Throughout the experiments no spontaneous mutations were detected for the recipient strain

Table 3
Features of isolated IncP-1 plasmids from Ria de Aveiro lagoon.

Plasmid	Selective marker	Sampling site	Aquatic environment	IncP-1 subgroup	Resistance patterns
pMLUA1	TET	CS	SML	ε	TET
pMLUA2	TET	CS	SML	β	TET; STX
pMLUA3	TET	CC	SML	ε	TET
pMLUA4	TET	CC	SML	ε	TET
pMLUA5	Hg	CC	SML	β	Hg
pMLUA6	Hg	CC	UW	β	Hg
pMLUA7	Hg	CC	UW	β	Hg

TET: tetracycline; STX: trimethroprim-sulfamethoxazole; Hg: mercury; CS: cais do Sporting; CC: cais do Chegado SML: surface sea microlayer; UW; underlying waters.

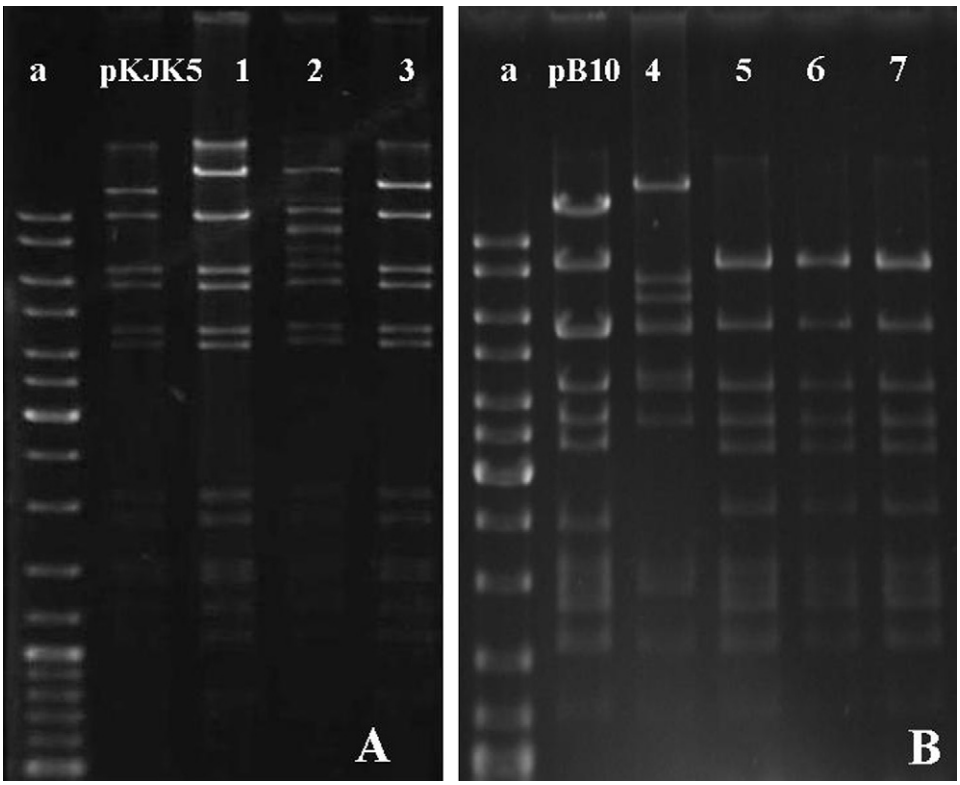

Fig. 1. Illustration of agarose gels of NotI-digested plasmids from ε (A) and β (B) IncP-1 subgroups. Lanes identified as pKJK5 and pB10 concern digests of phylogenetically close plasmids from ε and β IncP-1 plasmids, respectively; lanes 1–3 concern plasmid DNA digests of pMLUA1, pMLUA3 and pMLUA4, and lanes 4–7 concern digests of pMLUA2 and pMLUA5-7, respectively; lane *a* shows molecular size marker (Gene ruler DNA Ladder Mix, Fermentas).

growing in the presence of tetracycline or mercury. The use of nutrient limiting conditions during matings, that mimic the natural aquatic environment, grants ecological relevance to the gene transfer frequencies and plasmids isolated (Dahlberg et al., 1997). Similar resistance plasmid isolation was attempted by Dahlberg and co-workers (1997), however, lower gene transfer frequencies, at least 100× lower, in biofilms and bulk water and much lower frequencies, about 1000× times, for air–water interface samples were obtained when comparing with conjugation frequencies in the estuary Ria de Aveiro. Tetracycline and mercury resistance transfer are likely to occur under the natural conditions of the estuary and plasmids isolated can be considered relevant for gene dissemination. CS and CC sites in Ria de Aveiro are mostly contaminated by port activities and industrial effluents release, respectively. In fact, the capture of mercury resistance was only possible in the CC site that has been highly contaminated by effluent discharges of a mercury cell chlor-alkali plant in the past decades (Pereira et al., 2009). Salinity and temperature for CC sampling site was 24.8 and 17 °C, respectively, whereas for CS salinity was 19.6 and temperature was 20.7 °C.

All of the obtained transconjugants were further probed for IncP, IncN, IncW and IncQ plasmids by PCR and hybridization. As a result 7 transconjugants were positive using

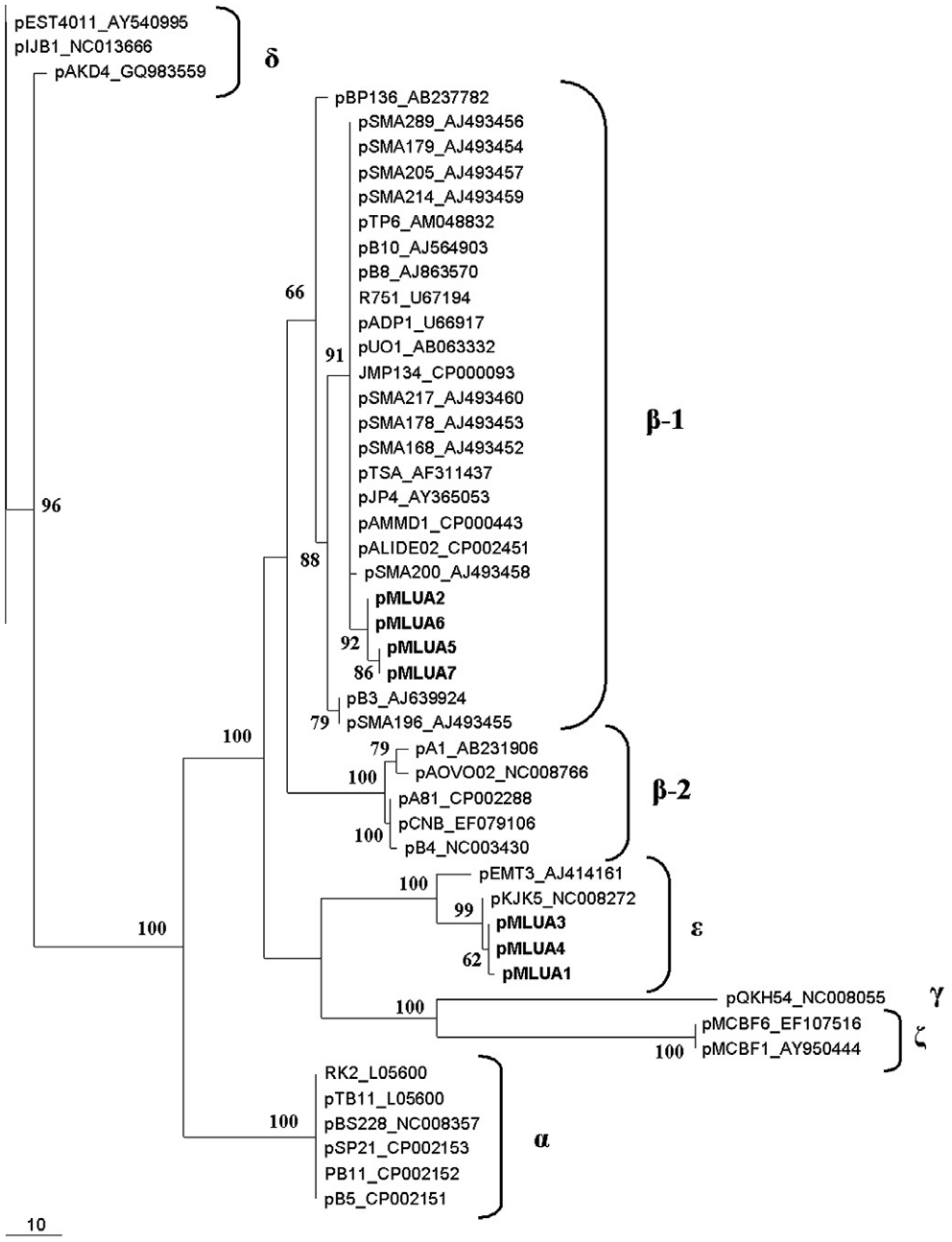

Fig. 2. Phylogeny of the amplified *tfrA* gene fragments of captured plasmids from Ria de Aveiro (pMLUA1-7) in relation with plasmids representing the known *trfA* gene diversity. Analysis was made based on multiple alignments (CLUSTALX) of the nucleotide sequences. PAUP4.0b Program was used to construct the parsimony tree. Bootstrap values based on 1000 replicates are shown as percentages. The distances between the fragments are indicated by the substitutions/site bar.

the *trfA* primer pairs (Table 2) and hence should carry IncP-1β, IncP-1α or IncP-1ε plasmids, since this primer pair amplifies replicon specific sequences from these three plasmids subgroups. The remaining transconjugants were negative for the presence of any of the sequences tested and hence could not be identified as BHR replicons. After, DNA isolation confirmed the presence of high molecular weight plasmid DNA (data not shown). The IncP-1 plasmid diversity was assessed by sequence analysis of the 281 bp *trfA* gene fragment. A multiple alignment was performed together with other sequences from previously described IncP-1 plasmids. All of the amplified gene fragments were almost identical (98% and 99%) to homologous regions of plasmids from IncP-1β and IncP-1ε groups. Five of the plasmids, named pMLUA1-5, were captured from SML samples (two from CS and the other from CC sampling site) and

were classified as β (pMLUA2 and pMLUA5) and ε (pMLUA1, pMLUA3 and pMLUA4) IncP-1 plasmids. The other plasmids, pMLUA6 and pMLUA7, were from UW samples collected at CC site and identified as IncP-1β plasmids. Also, and as seen in Table 3, tetracycline resistance was the selective marker for 4 plasmids and mercury for the other 3.

Plasmid DNA restriction analysis showed similar banding patterns between plasmids from the same subgroups (Fig. 1A and B), a result of the highly conserved plasmid backbones. However, differences were seen. Plasmids pMLUA1 and pMLUA3-4, assigned to the IncP-1ε subgroup, showed differences between each other in higher molecular weight bands and also when compared with pKJK5 revealing genetic diversity of tetracycline resistant IncP-1ε plasmids in the estuary. When analyzing the restriction patterns of the plasmids assigned to IncP-1β subgroup, banding patterns were less diverse. Plasmids pMLUA5-7 apparently show identical profiles, while pMLUA2 presents a unique restriction pattern (Fig. 1, lanes 4–7), even when compared with the model plasmid pB10.

Regarding susceptibility testing mercury susceptibility was seen for the transformants carrying tetracycline resistant plasmids (plasmids pMLUA1-4) while transformants carrying plasmids isolated by use of mercury resistance were sensible to this antibiotic. Apart from this, only pMLUA2 showed an additional resistance to the combination trimethoprim-sulfamethoxazole (Table 3).

We analyzed the phylogeny of the amplified 281 bp gene fragment of *trfA*. For that, the nucleotide sequences from pMLUA plasmids were compared with 41 sequences retrieved from GenBank corresponding to plasmids belonging to α, β, ε, γ, δ and ζ IncP-1 subgroups and reflecting the known diversities of the *trfA* gene available up until now. Among them, 29 have their complete DNA backbone sequenced and the rest only have partial sequences available. Parsimony tree constructed (Fig. 2) showed 7 different clades, representing the already described IncP-1 subgroups (Bahl et al., 2009 and Norberg et al., 2011). Plasmids pMLUA2 and pMLUA5-7 were grouped within the β-1 clade, being closer to pJP4 or pB10 like sequences than from the plasmids pB3, pSMA196 and pBP136, affiliated also in this branch. Among them, pMLUA2 and pMLUA6, and pMLUA5 and pMLUA7 shared 100% identity in the *trfA* gene fragment sequence. Regarding pMLUA2 and pMLUA6 (Table 3), they were captured in different sampling sites of Ria de Aveiro and were from SML and UW environments, respectively, suggesting that they are widespread along the estuarine bacterial communities. As for plasmids pMLUA5 and pMLUA7, the same resistance pattern and the fact that they present identical restriction profiles suggest that they might represent the same plasmid.

Regarding pMLUA1, pMLUA3 and pMLUA4, they grouped within the ε branch and are phylogenetically closer to pKJK5 than to pEMT3 (Fig. 2), the only representatives of this branch, up until now. They all present some genetic variability as seen by restriction analysis (Fig. 1) even though no differences were found among the susceptibility tests carried out. As hypothesized for IncP-1 plasmids in general (Norberg et al., 2011), the genetic variability seen for these estuarine plasmids can be due to specific plasmid/host coevolutions and/or to multiple selective pressure forces present along the estuary.

It is known that IncP-1 plasmids diversity has been biased by plasmid capture methods, source environment, use of selective markers and PCR probing (Smalla and Sobecky, 2002). This can easily be perceived by recent plasmid massive sequencing that is providing the description of new IncP-1 replicons, making up new clades, such as IncP-1ζ from marine biofilm or the IncP-1γ-(expanded) (Norberg et al., 2011; Stenger and Lee, 2011). The work presented here shows that new molecular variants of IncP-1β and IncP-1ε plasmids are present in estuarine waters and, together with the recent finding that a new IncP-1 subgroup (ζ) is present in marine biofilms (Norberg et al., 2011), shows that natural aquatic environments may represent a rich source of these BHR plasmids. Regarding the ε subgroup only pKJK5 from manure (Bahl et al., 2007), pEMT3 from a soil mesocosm experiment (Top et al., 1995), and plasmids from piggery manure (Binh et al., 2008) make up the known replicons within this group. The isolation of three apparently different IncP-1ε plasmids occurring in an estuary will further support the characterization of this topical subgroup justifying further efforts for their full characterization. In fact, the sequence determination of the IncP-1 plasmids isolated in this investigation is currently underway.

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References

Agogué, H., Casamayor, E.O., Joux, F., Obernosterer, I., Dupuy, C., Lantoine, F., Catala, P., Weinbauer, M.G., Reinthaler, T., Herndl, G.J., Lebaron, P., 2004. Comparison of samplers for the biological characterization of the sea surface microlayer. Limnol. Oceanogr. Methods 2, 213–225.

Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, J., Zhang, Z., Miller, W., Lipman, D.J., 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 25, 3389–3402.

Amann, R.I., Ludwig, W., Schleifer, K.H., 1995. Phylogenetic identification and in situ detection of individual microbial cells without cultivation. Microbiol. Rev. 59, 143–169.

Bahl, M.I., Hansen, L.H., Goesmann, A., Sorensen, S.J., 2007. The multiple antibiotic resistance IncP-1 plasmid pKJK5 isolated from a soil environment is phylogenetically divergent from members of the previously established alpha, beta and delta subgroups. Plasmid 58, 31–43.

Bahl, M.I., Burmølle, M., Meisner, A., Hansen, L.H., Sørensen, S.H., 2009. All IncP-1 plasmid subgroups, including the novel e subgroup, are prevalent in the influent of a Danish wastewater treatment plant. Plasmid 62, 34–139.

Bale, M.J., Day, M.J., Fry, J.C., 1988. Novel method for studying plasmid transfer in undisturbed river epilithon. Appl. Environ. Microbiol. 54, 2756–2758.

Binh, C.T.T., Heuer, H., Kaupenjohann, M., Smalla, K., 2008. Piggery manure used for soil fertilization is a reservoir for transferable antibiotic resistance plasmids. FEMS Microbiol. Ecol., 1–13.

Cunliffe, M., Upstill-Goddard, R.C., Murrell, J.C., 2010. Microbiology of aquatic surface microlayers. FEMS Microbiol Rev 35 (2), 233–243.

Dahlberg, C., Linberg, C., Torsvik, V.L., Hermansson, M., 1997. Conjugative plasmids isolated from bacteria in marine environments show various degrees of homology to each other and are not closely related to well-characterized plasmids. Appl. Environ. Microbiol. 63 (12), 4692–4697.

Dahlberg, C., Bergstrom, M., Hermansson, M., 1998. In situ detection of high levels of horizontal plasmid transfer in marine bacterial communities. Appl. Environ. Microbiol. 64 (7), 2670–2675.

Droge, M., Puhler, A., Selbitschka, W., 2000. Phenotypic and molecular characterization of conjugative antibiotic resistance plasmids isolated from bacterial communities of activated sludge. Mol. Gen. Genet. 263, 471–482.

Ducklow, H.W., Carlson, C.A., 1992. Oceanic bacterial production. Adv. Microbiol. Ecol., 113–181.

Francisco, D.E., Mah, R.A., Rabin, A.C., 1973. Acridine orange epifluorescent technique for counting bacteria in natural water samples. Trans. Am. Microsc. Soc. 92, 416–421.

Frost, L.S., Leplae, R., Summers, A.O., Toussaint, A., 2005. Mobile genetic elements: the agents of open source evolution. Nature Rev. Microbiol. 3, 722–732.

Gotz, A., Pukall, R., Smit, E., Tietze, E., Prager, R., Tschape, H., Van Elsas, J.D., Smalla, K., 1996. Detection and characterization of broad-host-range plasmids in environmental bacteria by PCR. Appl. Environ. Microbiol. 62 (7), 2621–2628.

Heinemann, J.A., Sprague, G.F., 1989. Bacterial conjugative plasmids mobilize DNA transfer between bacteria and yeast. Nature 340, 205–209.

Henriques, I.S., Fonseca, F., Alves, A., Saavedra, M.J., Correia, A., 2006. Occurrence and diversity of integrons and beta-lactamase genes among ampicillin resistant isolates from estuarine waters. Res. Microbiol. 157, 938–947.

Hermansson, M., Jones, G.W., Kjelleberg, S., 1987. Frequency of antibiotic and heavy metal resistance, pigmentation, and plasmids in bacteria of the marine air-water interface. Appl. Environ. Microbiol. 53 (10), 2338–2342.

Heuer, H., Krogerrecklenfort, E., Wellington, E.M.H., Egan, S., van Elsas, J.D., van Overbeek, L., Collard, J.-M., Guillaume, G., Karagouni, A.D., Nikolakopoulou, T.L., Smalla, K., 2002. Gentamicin resistance genes in environmental bacteria: prevalence and transfer. FEMS Microbiol. Ecol. 42, 289–302.

Jones, G.W., Baines, L., Genthner, F.J., 1991. Heterotrophic bacteria of the freshwater neuston and their ability to act as plasmid recipients under nutrient deprived conditions. Microb. Ecol. 22, 15–25.

Kreps, S., Ferino, F., Mosrin, C., Gerits, J., Mergeay, M., Thuriaux, P., 1990. Conjugative transfer and autonomous replication of the promiscuous IncQ plasmid in the cyanobacterium Synechocystis PCC 6803. Mol. Gen. Genet. 221, 129–133.

Laroche, E., Pawlak, B., Berthe1, T., Skurnik, D., Petit, F., 2007. Occurrence of antibiotic resistance and class1, 2 and 3 integrons in *Escherichia coli* isolated froma densely populated estuary (Seine, France). FEMS Microbiol. Lett. 271, 147–161.

Moura, A., Henriques, I., Smalla, K., Correia, A., 2010. Wastewater bacterial communities bring together broad-host range plasmids, integrons and a wide diversity of uncharacterized gene cassettes. Res. Microbiol. 161 (1), 58–66.

Norberg, P., Bergström, M., Jethava, V., Hermansson, D., 2011. The IncP-1 plasmid backbone adapts to different host bacterial species and evolves through homologous recombination. Nature Communications 2, 268.

Pereira, M.E., Lillebo, A.I., Pato, P., Válega, M., Coelho, J.P., Lopes, C.B., Rodrigues, S., Cachada, A., Otero, M., Pardal, M.A., Duarte, A.C., 2009. Mercury pollution in Ria de Aveiro (Portugal): a review of the system assessment. Environ. Monit. Assess. 155, 39–49.

Performance Standards for Antimicrobial Susceptibility Testing. Twentieth informational supplement. CLSI document M100-S20, Clinical and Laboratory Standard Institute, Wayne, 2010.

Sambrook, J., Fritsch, E.F., Maniatis, T., 1989. Molecular Cloning, 2nd ed. Cold Spring Harbor Laboratory Press, New York.

Santos, A.L., Mendes, C., Gomes, N.C.M., Henriques, I., Correia, A., Almeida, A., Cunha, A., 2009. Short-term variability of abundance, diversity and activity of estuarine bacterioneuston and bacterioplankton. J. Plankton Res 31 (12), 1545–1555.

Schmidt, A.S., Bruun, M.S., Dalsgaard, I., Larsen, J.L., 2001. Incidence, distribution, and spread of tetracycline resistance determinants and integron-associated antibiotic resistance genes among motile aeromonads from a fish farming environment. Appl. Environ. Microbiol. 67, 5675–5682.

Smalla, K., Sobecky, P.A., 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., Haines, A.S., Jones, K., Krogerrecklenfort, E., Heuer, H., Schloter, M., Thomas, C.M., 2006. Increased abundance of IncP-1β plasmids and mercury resistance genes in mercury-polluted river sediments: first discovery of IncP-1β plasmids with a complex *mer* transposon as the sole accessory element. Appl. Environ. Microbiol. 72 (11), 7253–7259.

Sorensen, S.J., Bailey, M., Hansen, L.H., Kroer, N., Wuertz, S., 2005. Studying plasmid horizontal transfer in situ : a critical review. Nat. Rev. Microbiol. 3, 700–710.

Stenger, D.C., Lee, M.W., 2011. Phylogeny of replication initiator protein TrfA reveals a highly divergent clade of incompatibility group P1 plasmids. Appl. Env. Microbiol. 77 (7), 2522–2526.

Swofford, D.L., 2002. PAUP: Phylogenetic analysis using parsimony (and other methods). Version 4. Sinaur Associates, Sunderland, Massachusetts.

Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F., Higgins, D.G., 1997. The CLUSTAL_X windows interface. flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. 25, 4876–4882.

Top, E.M., Smet, I., Verstraete, W., Dijkmans, R., Mergeay, M., 1994. Exogenous isolation of mobilizing plasmids from polluted soils and sludges. Appl. Environ. Microbiol. 60 (3), 831–839.

Top, E.M., Holben, W.E., Forney, L.J., 1995. Characterization of diverse 2, 4-dichlorophenoxyacetic acid-Degradative plasmids isolated from soil by complementation. Appl. Environ. Microbiol. 61 (5), 1691–1698.

Trieu-Cuot, P., Carlier, C., Martin, P., Courvalin, P., 1987. Plasmid transfer by conjugation from *Escherichia coil* to gram-positive bacteria. FEMS Microbiol. Lett. 48, 289–294.