Universidade de Aveiro Departamento de Biologia
2010

Beatriz Lázaro Pinto

Plasmídeos com Gama Alargada de Hospedeiros em Ambientes Estuarinos

Broad Host Range Plasmids in Estuarine Environments

Universidade de Aveiro Departamento de Biologia
2010

Beatriz Lázaro Pinto

Plasmídeos com Gama Alargada de Hospedeiros em Ambientes Estuarinos

Broad Host Range Plasmids in Estuarine Environments

dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biotecnologia, realizada sob a orientação científica da Doutora Cláudia Oliveira, Investigadora em Pós-Doutoramento do CESAM e do Professor Doutor António Correia, Professor Catedrático do Departamento de Biologia da Universidade de Aveiro

o júri

presidente

Prof. Doutora Maria da Conceição Lopes Vieira dos Santos
professora associada com agregação do Departamento de Biologia da Universidade de Aveiro

Prof. Doutora Paula Maria Lima e Castro
professora auxiliar da Escola Superior de Biotecnologia da Universidade Católica Portuguesa

Doutora Cláudia Sofia Soares de Oliveira
Investigadora em Pós-Doutoramento do CESAM

Prof. Doutor António Carlos Matias Correia
professor Catedrático do Departamento de Biologia da Universidade de Aveiro

agradecimentos

Em primeiro lugar quero agradecer à minha orientadora, Doutora Cláudia Oliveira, pela orientação, pelo grande apoio prestado e por todos os sacrifícios que fez para ajudar para a concretização deste trabalho. Sinceramente muito obrigada por tudo!
Não poderia deixar de agradecer ao Professor António Correia pela oportunidade de desenvolver este trabalho no seu laboratório e pelo seu contributo e apoio ao longo deste tempo.
Agradeço a todos os companheiros de laboratório, e especialmente à Doutora Isabel Henriques e à Juliana Nina de Azevedo pela ajuda e disponibilidade prestada.
Finalmente devo agradecer à Universidade de León (Espanha) por tornar possível a minha estadia em Aveiro.

palavras-chave

Camada superficial do mar, Trasferência horizontal de genes, Análise filogenética, Plasmídeos IncP-1, Gene *trfA*

resumo

A transferência horizontal de genes permite a adaptação microbiana a nichos especiais, dos quais dois bons exemplos são a camada superficial do mar e a coluna de água. Os plasmídeos com gama alargada de hospedeiros (BHR), responsáveis pelo fluxo de material genético entre cromossomas bacterianos (inclusivamente entre microorganismos muito afastados filogeneticamente) têm um papel essencial na evolução das comunidades microbianas. Entre eles, os plasmídeos pertencendo ao grupo de incompatibilidade IncP-1 têm um interesse especial por causa da sua extraordinária flexibilidade na iniciação da replicação e da estabilidade na sua manutenção numa ampla gama de hospedeiros. Estes elementos genéticos móveis, além de constituírem ferramentas úteis na engenharia genética, representam uma grande fonte de genes que codificam para características tão significativas como a resistência a antibióticos e a degradação de xenobióticos. No entanto, a diversidade nesta família de plasmídeos BHR tem sido subestimada até agora: actualmente sabe-se da existência de cinco subgrupos divergentes, mas embora alguns plamídeos modelo tenham sido estudados ao detalhe, ainda há muito para investigar.

Em trabalhos anteriores na Ria de Aveiro (costa NW de Portugal), foi feita a captura exógena de plasmídeos bem como o isolamento de bactérias potencialmente hospedeiras de plasmídeos endógenos. Neste trabalho, sequências de nucleótidos específicas foram amplificadas mediante reacções em cadeia da polimerase para determinar a presença de plasmídeos BHR. Os sete plasmídeos IncP-1 detectados, foram em primeiro lugarfilogeneticamente estudados. O alinhamento das sequências de nucleótidos de 281 pb que foram amplificadas e que correspondem a um fragmento do gene *trf*A (que codifica para uma proteína do início da replicaçao) sugeriu o estabelecimento de dois novos *clusters* situados filogeneticamente em dois subgrupos diferentes de IncP-1: IncP-1β e o recentemente descrito IncP-1ε. Estes constituem os primeiros replicões IncP-1 provenientes de ambientes estuarinos a serem detectados e isolados. De seguida, uma comparação e caracterização preliminar genética e fenotípica foi realizada com os plasmídeos purificados, considerando a descrição já conhecida dos dois plasmídeos arquétipos evolutivamente mais próximos, pB10 e pKJK5. Assim, as análises de fragmentos de restrição, determinação da inibição do crescimento do hospedeiro na presença de mercúrio e ensaios de resistência a diferentes antibióticos ajudaram a compreender o elevado interesse que recai nestes plasmídeos revelando a diversidade fenotípica e genotípica. Uma completa descrição de qualquer destes novos plasmídeos pode ter una enorme importância ecológica, evolutiva e biotecnológica, incrementada pela sua procedência dum ambiente não clínico. Portanto este trabalho justifica um estudo em maior profundidade destes replicões promíscuos.

keywords

Sea surface microlayer, Horizontal gene transfer, Phylogenetic analysis, IncP-1 plasmids, *trfA* gene

abstract

The horizontal gene transfer allows microbial adaptation to special niches, from which the sea-surface microlayer or the subsurface waters in estuarine environments might be good examples. Broad host range plasmids, responsible for the reshuffling of genetic material between bacterial chromosomes (even amongst distantly related microorganims), play an essential role on the evolution and diversity of microbial communities. Among them, the incompatibility group IncP-1 plasmids have a special interest due to their extraordinary flexibility in the replication initiation and stable maintenance in such a wide spectrum of hosts. These mobile genetic elements, in addition to the helpful genetic engineering tools they mean, represent a great source of potentially useful genes encoding for traits as significant as antibiotic resistance or xenobiotic degradation. Nevertheless, the diversity of this family of BHR plasmids has been underestimated until recently: it is currently known to have five divergent sub-groups, but although some prototype plasmids have been studied in great detail, there is still much left to research.

In previous investigations exogenous plasmid capture was carried out as well as putative endogenous plasmid bacterial hosts isolated in the *Ria de Aveiro* lagoon (NW coast of Portugal). In this work, polymerase chain reactions were developed to amplify specific nucleotide sequences and determine BHR plasmids presence. From a bioinformatical approach, the seven IncP-1 plasmids detected were firstly phylogenetically studied. The alignment of the amplified 281 bp nucleotide sequences corresponding to a fragment of the replication initiation protein encoding gene *trfA* suggested the formation of two novel clusters belonging to two different IncP-1 plasmid subgroups: IncP-1β and the lately described IncP-1ε. Additionally, these represent the first estuarine IncP-1 replicons to be detected and isolated. Then a preliminary genetic and phenotypic comparison was performed with the purified plasmids, by taking into account the known description of the evolutionary closest models, pB10 and pKJK5. That way, restriction fragment analysis as well as antibiotic and mercury resistance determination assays helped to comprehend the high significance falling on the captured plasmids by revealing the genetic and phenotypic diversity. A whole description of any of these novel plasmids may have a huge ecological, evolutionary and biotechnological importance, even more due to its precedence from a non-clinical environment. Therefore this work justifies further studies on these promiscuous replicons.

Contents:

Index of tables

Index of figures

1. Introduction

1.1 Aquatic environments and microbial communities

The specific physical habitat or location of a microorganism is its microenvironment. There, the fluxes and gradients of required oxidants, reductants, and nutrients as well as waste products create a unique niche. However, the microorganisms can create their own microenvironments and niches. When microbial growth happens on surfaces, such as in freshwater and marine environments, biofilms are formed (Prescott et al., 2002b).

The physical frontier between the ocean and the atmosphere is the sea-surface microlayer (SML). This interface between the sea and the air is considered to be the around 1mm-top layer of the ocean (the layer of 10-100 µm-top was employed in this research). If compared with subsurface water (SSW) it is physical-chemically distinct and characteristically enriched with biogenic organic compounds, such as lipids, proteins and polysaccharides. That stratification makes the SML stable enough to exist at typical oceanic wind conditions (Wurl et al., 2009). The main point is that the SML could be made up of gel particles, with similar microbiological and biogeochemical characteristics. Some studies showed an enrichment of transparent exopolymer particles (TEP) in the SML (Wurl et al. 2008). TEPs are normally formed in surface waters from the coagulation of biogenic polysaccharides; chiefly those produced by phytoplankton, and are some of the most ubiquitous gel particles in the marine environment. They are critical in the formation of marine aggregates, acting as the binding matrix or ‘glue’ that holds the aggregate together (Verdugo et al., 2004).

The presence of the surface film and surface tension properties turn the SML into a unique habitat. Bacterial communities that are present in the SML are known as the bacterioneuston. Surface films also take place on all water bodies, marine, estuarine and freshwater, sometimes as visible slicks. The microbial communities established in the SSW are less dense and less structured, and constitute what is called the bacterioplancton (Cunliffe et al., 2009a).

The aggregation of bacterial cells seen in some studies (Franklin et al., 2005; Cunliffe et al., 2009a) most likely takes place in the SML, with bacterial cells attached to

the TEP-based gel particles reported by Wurl and Holmes (2008). It has been proposed that this enrichment on attached biofilm-growing cells is the main cause of the distinct properties of bacterioneuston. If, as suggested by Sieburth (1983), the SML is a gelatinous film in which prevails biofilm-growing cells, then biological processes that occur in bacterial communities and that are favored when bacterial cells are in close proximity, such as horizontal gene transfer (HGT) and quorum sensing, could be more frequent. The large specific microbiological diversity supposed to occur in this unique ecological niche supports a much broader molecular and functional diversities. Therefore, its subsequently possible biotechnological value makes its investigation interesting. Nonetheless, studies concerning the molecular microbial ecology of the SML started only recently (Cunliffe et al., 2009a).

1.2 Bacterial plasmids and their importance in shaping the properties of bacterial communities.

Nowadays there is evidence that plasmids and transposable elements can move genetic material between bacterial chromosomes to cause rapid changes in genomes and drastically alter phenotypes (Prescott et al., 2002a). The prokaryotic horizontal gene pool (HGP), defined as the mobile genetic elements (MGEs) and their encoded genes (Thomas, 2000b), plays a vital and essential role in the evolution and adaptation of individual microorganisms and microbial communities by generating genetic variations in bacteria (reviewed in Arber, 2000). Like that, the sequencing of complete bacterial genomes has clearly shown that a large proportion of their genetic diversity have been acquired by HGT and come from distantly related microorganisms. This evidence has been derived from nucleotide sequence comparisons as well as from atypical nucleotide composition (guanine + cytosine content) of large genomic regions or different patterns of codon usage of some genes (Ochman et al., 2000).

Huge efforts have been carried out to catalogue, characterize, and exploit the different aspects of the enormous biodiversity of microorganisms in every natural habitat. In contrast, there is an undeveloped understanding of the abundance, distribution and molecular diversity of MGEs extant in the HGP occurring in most marine, freshwater, and many terrestrial microbial communities (Sobecky, 2002). Probably that is due to the fact

that the importance of the HGT in bacterial persistence, diversity and evolution is less widely appreciated (Thomas, 2000b). The first time its significance was accepted match up with the moment when multiple antibiotic resistant pathogens emerged, since MGE played a primary role in the development and dissemination of antibiotic resistance genes and allowed bacterial populations to rapidly adapt to a strong selective pressure. Additionally, plasmid mediated conjugation is probably one of the most frequent mechanisms of HGT (Sota et al., 2008), which explain the particular interest falling on this kind of MGE.

Because of this reshuffling of genes between bacterial cells, populations and communities that makes plasmids remarkably influent in ecological processes, the study of plasmid ecology and transfer together with environmental microbiology and microbial ecology can provide essential information on plasmid evolution. The evolutionary change affects plasmids in the same way as their host bacteria so plasmids have been regarded as units of evolution. In addition, retrospective studies offer a means to infer evolutionary relationships directly from plasmids isolated from geographically similar or remote locations (Sobecky, 2002; Pickup et al., 1996).

Thus, since plasmids represent a large genetic resource of bacterial diversity, research on plasmid distribution, evolutionary relationships, and diversity of plasmids in relation to the natural selection pressures is required in order to comprehend the role they play in the flow of genetic information in natural bacterial communities (Dahlberg et al., 1997; Harada et al., 2006).

Furthermore, the HGT is also manifested in the “plasmids backbones” where recombination within homologous regions of closely related plasmids can be noticed. Since these regions are responsible for replication, maintenance, and plasmid transfer, these recombination events play a key role in increasing or reducing the host range of these plasmids. This could be imperative as much with regard to the species which could obtain new genetic traits as because it sets the genetic backgrounds from which plasmids can purchase new traits (Lipps et al., 2008).

Most of the actual knowledge of HGT is related to plasmids occurring in bacteria of medical and agricultural importance. These plasmids correspond to a limited collection of replicons that surely are not representative of the plasmid populations occurring in every environment.

Several plasmid types present in strains isolated from clinical environments can be also found in environmental samples such as manure slurries or marine sediments; however a large part of the plasmids isolated from bacterial strains of natural environments contain replication and incompatibility regions unrelated to those of the known groups of plasmids traditionally found associated to clinical environments. This justifies further evaluation of the prevalence, diversity and evolution of plasmids, especially in nonclinical environments (Thomas, 2000b; Sobecky, 2002; Gstalder et al., 2003).

1.3 The study of plasmids and its biotechnological significance

A vast pool of traits usually associated with plasmids rather than chromosomes confer advantages which allow bacterial communities to evolve, adapt to selective pressure and colonize certain habitats. Some examples of those traits are mentioned in the table I (Thomas, 2000b). These kind of habitats, among which a representative case is the SML, due to their extreme characteristics or to the way they bring diverse bacterial species together, may represent a good source of new plasmids or of old plasmid skeletons carrying new recombined segments. Ecological studies that contribute to a deeper knowledge on the distribution of different types of plasmids and their hosts are important in defining which environments are better to exploit, avoiding the inconvenient of blind extensive screenings.

The emergence of large multidrug resistance plasmids due to the strong selective pressure caused by antimicrobial chemotherapy is threatening to reverse development in the treatment of infectious diseases by augmenting human pathogens resistance to antimicrobial agents (Tauch et al., 2003; Tennstedt et al., 2005; Schlüter et al., 2007). Nevertheless, in addition to the clearly imperative significance of increasing the knowledge about resistance genes to clinically relevant antimicrobial drugs, there is also a huge interest in further study other kinds of functions carried by plasmids.

A large number of the xenobiotics that have been introduced to the environment at modern society are recalcitrant. Catabolic plasmids and their host strains have an enormous environmental and ecological significance due to their potential application for environmental biotechnology. Different catabolic pathways may be either chromosomally or plasmid-encoded (Sayler et al., 1990). However, ecologically, plasmid-encoded

pathways are beneficial because they provide genetically flexible systems and can be maintained in the population and transferred between bacterial species, without the need of technological support.

In addition to the genes that encode the degradation of man-made organic compounds, those responsible for the metabolism of naturally occurring pollutants are also assumed to be often located on plasmids or other mobile elements (Top et al., 2002).

Besides, since some accessory genes on these plasmids have no homologues and cannot be assigned a function, it is not known yet what phenotypes other than the well-known resistance and degradation functions may be encoded by certain plasmids (Sen et al., 2010). In this context, plasmids seem to be an extraordinary reservoir of molecular determinants of diverse functions and the technological potential of this reservoir deserves to be exploited.

Otherwise, plasmids are powerful vectors of recombinant DNA, allowing its spread in the environment. Its biotechnological importance in this respect lies in its utility as cloning vectors or vehicles of protein expression in different bacterial hosts. A complete knowledge of the distribution, maintenance, recombination, and conjugation of BHR plasmids is consequently essential to evaluate the risk associated with the release of recombinant DNA into the environment (Drönen et al., 1998; Gstalder et al., 2003).

As a conclusion it could be stated that plasmids may have increasingly important biotechnological applications, both respect to the large source of novel traits MGE represent and as useful tools in genetic engineering. Anyway, the first step in researching these potential utilities, as referred before, is further studying plasmid characterization, diversity and evolution.

Table I.Examples of traits usually associated with plasmids.

Phenotype encoded	***Plasmid example***	***References***
resistance to antibiotics	*pKJK5*	Bahl et al. (2007)
resistance to quaternary ammonium compounds (used as disinfectants)	*pB10*	Schluter et al. (2003)
resistance to heavy metals	*pQKH54*	Hill et al. (1992)
resistance to phages	*pCI658*	Forde et al. (1999)
responses to environmental stresses such as UV	*pKMIOI*	Kokjohn et al. (1994)
legume symbiosis	*pJB5JI*	Broughton et al. (1987)
tumor induction	*Ti*	Schell et al. (1977)
bacteriocins	*p4G6-6*	Neve et al. (1984)
pathogenicity determinants (hemolysins, toxins, adherence factors)	*pADAP*	Hurst et al. (2000)
degradation of man-made pollutants	*pEST4011*	Vedler et al. (2004)
production of toxins	*pTX14-1*	Faust et al. (1983)
morphological traits	*pPL376*	Hundle et al. (1994)
proteins involved in diverse metabolic functions	*pTF5*	Dominy et al. (1997)
bacteria to bacteria communication or bacteria-host interactions	*pWR100.*	Bernardini et al. (1989)

1.4 Plasmid biology

1.4.1 Molecular structure of plasmids

Plasmids can be considered as being made up of two distinct regions: the ‘‘backbone” region, which encodes functions involved in replication, transfer, and

maintenance and control of the plasmid; and the ‘‘accessory” region consisting of genes that may confer specific beneficial traits to the bacterial host (Thomas, 2000a).

In any case, plasmids are not at all static genomes, but instead are plastic genetic mosaics that have evolved over time through the iterative acquisition of various transposons, integrons, gene cassettes, genomic islands and insertion sequences, which are almost always associated with accessory genes, placing them in one or two sites between essential DNA fragments i.e., between *oriV* (the origin of vegetative replication), and *trfA* (a gene for plasmid replication) and/or between the two transfer operons that encode mating-pair formation and plasmid transfer *trb* and *tra*. Plasmids with almost identical backbones can have entirely different accessory genes (Lipps et al., 2008; Sen et al., 2010; Schlüter et al., 2007). Another essential gene that should be named is *oriT*, the origin of plasmid transfer. *Mob* proteins in mobilizable plasmids are necessary to convert the plasmids into the transferable form.

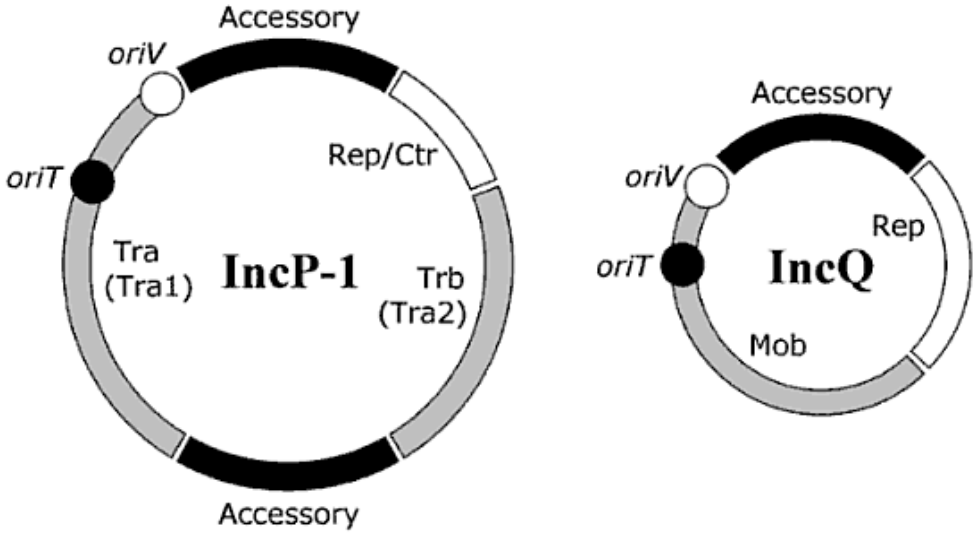


Fig 1. Schematical structures of IncP-1 and IncQ plasmids. The white area represents the regions for plasmid replication and control; the grey one, those genes involved in plasmid transfer (or mobilization); black symbolize the accessory genes (Lipps, 2008).

However, plasmids without such functional elements, these accessory elements, have also been described (Kamachi et al., 2006).

In other respects, plasmids are known to have complex recombinational histories. Some survival functions depend on just one genetic locus, but most depend on at least

two. The first sorts of modules to emerge would probably have consisted of interdependent functions but, at the same time, independent but mutually beneficial functions probably became clustered, as reviewed in Thomas (2000a).

1.4.2 Incompatibility groups and BHR plasmids.

Plasmid classification has been originally based in a property inherent in them, which is in addition a manifestation of relatedness (since it is the sharing of common elements related to plasmid control of replication and partition). Incompatibility is the inability of two plasmids to be maintained stably in the same cell line (Couturier et al., 1988). Two plasmids which cannot stay in the same cell belong to the same incompatibility group.

Four of these incompatibility groups are considered to be broad-host-range (BHR) plasmids. In spite of the difficulty of determining the host range of a plasmid, as transfer can be tested experimentally only between hosts representing a minute fraction of the bacterial world, Szpirer and co-workers (1999) proposed, in an attempt to define BHR, that plasmids which can transfer and replicate in bacterial species from at least two branches of the Proteobacteria should be viewed as BHR plasmids. Then the conjugative IncP, IncN and IncW, plasmids and the mobilizable IncQ plasmids are regarded as having BHR (Gstalder et al., 2003).

The role of BHR plasmids is remarkable when speaking about interspecies gene exchange since they are most likely the single intermediaries of HGT between distantly related bacterial hosts. IncQ plasmids have the broadest host-range of all of them in gram negative bacteria, followed of those of the group IncP-1 (based in the *Pseudomonas* classification system). Apart from spreading genes across taxonomically distant species by conjugative transfer, BHR plasmids can partially integrate into the recipient chromosome, or mobilize non-conjugative vectors with a wider host range (Lipps, 2008). They can also retromobilize or retrotransfer i.e., capturing genes or non-self-transmissible plasmids from host in which they cannot replicate (Szpirer et al., 1999). All these reasons make research about BHR plasmids especially interesting.

1.5 The group of IncP-1 plasmids

The distinctive characteristic of low copy number plasmids belonging to the incompatibility group IncP-1 is the central control operon coding for at least three global regulators. It gives them flexibility in the replication initiation, multiple stability mechanisms and coordinates regulation of all plasmid backbone functions, providing them with enormous adaptability and stable maintenance in such a wide spectrum of hosts. This ability of replicating and being stably maintained in almost all Gram-negative bacteria as well as being transferred by conjugation to Gram-positive bacteria, yeasts and eukaryotic cell lines justifies the particular interest that fall on IncP-1 plasmids (Adamczyk et al., 2003; Waters, 2001; Harada et al., 2006).

In spite of the high infection transfer rates the IncP-1 plasmids have, horizontal transfer is not enough for them to be maintained as genetic parasites, as they are a burden to their host, so that they need to carry at least occasionally advantageous traits to be maintained in bacterial communities. Consequently, the majority of the IncP-1 plasmids have large regions with acquired genes encoding traits which occasionally might augment the fitness of the bacterial host (Bergstrom et al., 2000).

It appears that the IncP-1 backbone can either carry antibiotic-resistance determinants or degradative operons but to the best of our knowledge, a plasmid that carries both types of genes (antibiotic-resistance and degradative genes) has not yet been identified (Schlüter et al., 2003).

Regardless of the large range of accessory elements among IncP-1 plasmids, they may be phylogenetically grouped into a small number of distinct subgroups based on their backbone sequences. As a result, during the last years, phylogenetic analysis of the 281bp-region of the replication gene *trfA*, as well as a few other essential backbone genes regions, has allowed the IncP-1 plasmid group to be classified into five main phylogenetic sub-groups: IncP-1α, -β, -γ, -δ and -ε (Sen et al., 2010; Bahl et al., 2009).

The α and β subgroups constitute the two first recognized subgroups and have been extensively studied. The δ and γ subgroups were defined only based on a phylogenetic analysis of their backbone genes. Later, three IncP-1 δ and one IncP-1 γ plasmids were completely sequenced and described (Xia et al., 1998; Vedler et al., 2004; Sen et al., 2010; Hill et al., 1992). The presence of a fifth subgroup, ε, was recently

suggested by Bahl (2007) consisting of pEMT3 (from which just a fragment is known) and pKJK5 that has been completely sequenced (Gstalder et al., 2003; Top et al., 1995; Bahl et al., 2007).

In the table II some information about the completly sequenced IncP-1 plasmids that represent the full known diversity within this 281 bp-region of the *trfA* gene is shown.

Table II. Completely sequenced IncP-1 plasmids representing the whole phylogeny of this incompatibility group of plasmids according to Bahl et colleagues (2009).

Subgroup	Plasmid names	Encoded traits	Isolation technique	Isolation environment	Host	Acession number	Reference(s)
α	RK2 (IncP-1α prototype)	Resistance to ampicilin (AMP), kanamycin (KAN) and tetracycline (TET)	Endogenous	Burns unit, Birmingham Accident, Hospital, Birmingham, UK (1969)	*Escherichia coli*	NC_001621	Datta et al. (1971)
α	pTB11	Resistance to ampicilin (AMP), cefaclor monohydrate (CEC), cefuroxime sodium (CMX), gentamicin (GEN), kanamycin (KAN), streptomycin (STR), spectinomycin, tetracycline (TET), tobramycin sulfate (TOB)	Exogenous (in *Pseudoalteromonas)*	Wastewater treatment plant, Bielefeld-Heepen, Germany (2002)	*Uncultured bacterium*	NC_006352.1	Tennstedt et al. (2005)
α	pBS228	Resistance to tetracycline (TET), streptomycin (STR), trimethoprim (TMP), ampicilin (AMP), spectinomycin, streptothricin	Endogenous	Waste water from factory, Moscow, Russia	*Pseudomonas aeruginosa*	NC_008357	Haines et al. (2007)
β	R751 (IncP-1β prototype)	Resistance to trimethoprim (TMP)	Endogenous	St. Thomas's hospital, London, UK (1972)	*Klebsiella aerogenes (Enterobacter aerogenes)*	NC_001735	Jobanputra et al. (1974)
β	pJP4	Resistance to 2,4-D3-Chlorobenzoate	Endogenous	(Australia)	*Ralstonia eutropha JMP134*	NC_005912	Don and Pemberton (1981)
β	pADP1	Resistance to Atrazine (ATR)	Endogenous	Herbicide spill site/agricultural soil, Little Falls, Minnesota, USA	*Pseudomonas sp. Strain ADP*	NC_004956	Mandelbaum et al. (1993) and Mandelbaum et al. (1995)
β	pUO1	Resistance to mercury ions and haloacetates	Endogenous	Industrial wastewater, Japan	*Delftia acidovorans strain B*	NC_005088	Sota et al. (2002)
β	pB4	Resistance to streptomycin (STR), spectinomycin, ampicillin (AMP) and sulfonamides (SA)	Exogenous (in *Pseudomonas* sp.)	Activated sludge from municipal wastewater treatment plant, Braunschweig, Germany	*Uncultured bacterium*	NC_003430	Tauch et al. (2003)
β	pB10	Resistance to mercury ions, quaternary ammonium compounds and disinfectants, beta-lactam antibiotics, amoxicillin, streptomycin (STR), sulfonamides (SA) and tetracycline (TET)	Exogenous (in *Pseudomonas* sp.)	Activated sludge from municipal wastewater treatment plant, Braunschweig, Germany	*Uncultured bacterium*	NC_004840	Schluter et al. (2003)
β	pA81	PCBs degradation	Endogenous	Polychlorinated biphenyl-contaminted soil, Zamberk, Czech Republic	*Achromobacter xylosoxidans*	NC_006830	Jencova et al. (2004)

Subgroup	*Plasmid names*	*Encoded traits*	*Isolation technique*	*Isolation environment*	*Host*	*Acession number*	*Reference(s)*
β	pB3	Resistance to streptomycin (STR), spectinomycin, ampicillin (AMP), sulfonamides (SA), chloramphenicol (CAP) and tetracycline (TET)	Exogenous (in *Pseudomonas* sp.)	Activated sludge from municipal wastewater treatment plant, Braunschweig, Germany	*Uncultured bacterium*	NC_006388	Heuer et al. (2004)
β	pB8	Resistance to streptomycin (STR), spectinomycin and sulfonamide (SA)	Exogenous (in *Pseudomonas* sp.)	Activated sludge from municipal wastewater treatment plant, Braunschweig, Germany	*Uncultured bacterium*	NC_007502	Schluter et al. (2005)
β	pTP6	Resistance to mercury ions	Exogenous-triparental (in Pseudomonas putida and Escherichia coli K12)	River sediment, river Nura (mercury contaminated), Kazakhstan (1999–2000)	*Uncultured bacterium*	NC_007680	Smalla et al. (2006)
β	pBP136	None	Endogenous	General hospital, Oita prefecture, Japan-2002	*Bordetella pertussis*	NC_008459	Kamachi et al. (2006)
β	pA1	None	Endogenous	Isolated from ditch sample/soil, Japan (1991)	*Sphingomonas sp.*	NC_007353	Harada et al. (2006)
δ	pIJB1	2,4-D, malonate degradation	Endogenous	Garden soil (UK)	*Burkholderia cepacia 2a*	NC_013666	Xia et al. (1998)
δ	pEST4011	2,4-D malonate degradation	Endogenous	Agricultural soil sample, Estonia	*Achromobacter xylosoxidans subsp. denitrificans EST4002*	NC_005793	Vedler et al. (2004)
δ	pAKD4	Resistance to mercury ions	Exogenous-bi/triparental (in *Pseudomonas putida* UWC1/ *P putida* KT2442, *Escherichia coli* CV601, *Cupriavidus necator* JMP228, and *Agrobacterium tumefaciens* UBAPF2)	Agricultural soil in Norway (1998)	*Uncultured bacterium*	GQ983559	Sen et al. (2010)
γ	pQKH54	Resistance to mercury ions	Exogenous (in *Escherichia coli*)	Epilithic bacteria, River Taff, South Wales, UK	*Uncultured bacterium*	NC_008055	Hill et al. (1992)
ε	pKJK5	Resistance to trimethoprim (TMP), tetracycline (TET); slight resistance to spectinomycin	Exogenous (in *Escherichia coli K12*)	Soil/manure, Taastrup, Denmark	*Uncultured bacterium*	NC_008272	Bahl et al. (2007)

1.5.1 Genetic and phenotypic characterization of IncP-1 plasmids

Plasmid comparison and characterization has often been studied through restriction fragment analyses. There are two important practical aspects to take into account: the first one is the quality of the plasmid DNA, because the analysis may be impaired by partial digest; the second one is the correct selection of the most suitable restriction endonuclease useful to generate appropriate restriction fragment length polymorphism (RFLP) patters, which depends on the plasmid sequence.

Studies concerning the heterologous DNA encoding certain phenotypic traits are of limited value for plasmid classification in its strict sense, since identical transposons can occur on different nonrelated plasmids. Nevertheless, research on these accessory functions is essential to determine the ecological role of plasmids and improve the knowledge about its evolution as a consequence of the responses to environmental stresses. Consequently, the great importance of heterologous DNA makes it be included in the general process for plasmid classification (Tsäpe, 1994; Thomas, 2000b).

1.6 Methodological considerations

Studies in such specific environments as is the case of the SML suppose to play special attention to some difficulties they involve, by establishing singular strategies and methodological approaches as appropriate as possible. The sample collection obtained may vary accordingly to the sampling method and strategy used and that has to be considered. That is the case, for instance, of sampling the SML or the SSW.

1.6.1 Methods of sample harvest

The SSW and even more the SML in the natural environment are submitted to temporal variations at a given place. This temporal and spatial variability of the samples has to be taken into account when analyzing the presence of plasmids in bacteria from environments such as surface waters. The quantity of samples should be a compromise between statistical requirements and practical considerations; the time between sampling, sample processing and analysis should be on the shortest possible way to circumvent shifts in the microbial populations and their activity and the number of parameters to be

analyzed should be the highest to increase the impact of spatial and temporal SML heterogeneity on the final results. In addition, natural turbulences may disturb the sampling work. The objective is to attain the closest biological composition of the sample to the original distribution in the SML that can be possible (Agogué et al., 2004; Thomas, 2000b)

The SML sampling techniques used may be therefore the key issue, since different sampling approaches will represent different depths of the SML, thus influencing the samples composition collected. Also, some methods may be biased toward specific cell types, determining this way the results of the research. Consequently, it is essential to carry out a complete comparison of all SML sampling methods utilized, especially when using molecular tools to analyze the microbial ecology of these samples (Cunliffe et al., 2009a).

The most common samplers are the mesh screens (MS), glass plates (GP), hydrophobic (PTFE, polytetrafluoroethylene) and hydrophilic (PC, polycarbonate) membranes (Cunliffe et al., 2009b). Employing these methods bacterial community structures collected in the SML and in SSW can be compared. However, the question of the most suitable method of sampling the SML is far from being answered (Agogué et al., 2004; Cunliffe et al., 2009b).

1.6.2 Methods to carry out the plasmid isolation

The determination of the distribution of plasmids in microbial communities has traditionally been carried out with the initial cultivation of bacterial hosts, with or without the use of selective media types, for the screening and confirmation of plasmids, which is the so called *endogenous isolation* (Sobecky, 2002).

On the flipside, the small proportion of bacteria accessible to cultivation techniques explains the lack of information on the incidence and abundance of plasmids in the natural environment. In addition, culturable bacteria are known to respond to environmental stress by the formation of viable but nonculturable cells (Roszack et al., 1987).

Conventionally numerous procedures employing a variety of cell lysis and extraction conditions and different commercial extraction kits have been used in the plasmid isolation from culturable bacterial hosts; more recently, methods to isolate plasmids independently from the culturability of their original hosts have become available

(Bale et al., 1987). This alternative method, known as *exogenous isolation*, consists on "capturing" plasmids directly from the bacterial community via mating with a selectable recipient strain. In its two variations, named bi- and tri-parental isolation, it is required that the captured plasmid(s) either be self-transferable or mobilizable and subsequently able to replicate and express (selectable) plasmid-encoded genes in the recipient host (Sobecky, 2002).

Another way of plasmid detection has been established, the screening of environmental DNA. It consists on directly extracting the whole nucleic acids from environmental samples, combined with the amplification of specific plasmid sequences, which is facilitated by the development of DNA probes and PCR primers targeted to conserved plasmid replication and transfer regions (Sobecky, 2002; Thomas, 2000b). However this approach was not used in the present study so it won't be longer explained. This allows to improve the knowledge about the plasmid distribution in different places, an ecological approach that is, as referred before, essential to focus our research in potentially interesting environments where plasmid isolation strategies should be used.

1.6.3 Phenotypic traits for plasmids isolation.

One of the most toxic heavy metals that can be found in the environment, both due to geological processes and anthropogenic activities, is mercury. Molecular analysis has revealed a huge variety of genes encoding resistance to mercury ions which are reported to occur on a large range of plasmids belonging to various incompatibility groups, contributing that way to bacterial adaptability and reflecting at the same time environmental stresses (Smalla et al., 2006).

Since tetracycline was the first broad spectrum antibiotic with widespread usage in the treatment of diseases, resistance to tetracycline rapidly emerged. Presently, at least 17 different genes conferring tetracycline resistance are known and believed to come from many different ecosystems bacterial communities (Thomas, 2000b).

The known habitual presence of mercury or tetracycline resistance genes in plasmids leads to their use as selective markers to detect or capture plasmids from natural environments by means of the selective isolation of their bacterial hosts.

Additionally, the frequent correlation between mercury resistance genes and self-transmissible plasmids, as well as the common genetically link of antibiotic resistance genes to heavy-metal resistance genes, adaptation to mercury pollution may lead the spreading of antibiotic resistance genes throughout the bacterial community (Rasmussen et al., 1998). That adds significance to a deeper study on that kind of plasmids.

2. Objectives

Knowing that BHR plasmids may be gene carriers that allow bacterial adaptation to certain special conditions by carrying resistance, degradative or another kind of genes, it is our premise that these genes can have high biotechnological applications and those MGE should be consequently explored.

As referred before, ecological and evolutionary approaches are essential to understand plasmid influence on the flow of genetic information in bacterial communities. Knowledge on the distribution of different types of plasmids and their hosts is also important in defining which environments are more interesting to exploit and have more likely attractive results. The *Ria de Aveiro* lagoon, due to their definite characteristics and to their special microbial community, may show an important source of new plasmids or of old plasmid skeletons carrying new recombined segments.

Therefore, the current study aims to investigate the presence of BHR plasmids as well as the diversity of IncP-1 plasmid sequences in the plasmids captured from different places of the *Ria de Aveiro* lagoon and evaluate their phylogenetic relationship together with well characterized IncP-1 plasmids.

Another target of this thesis is a preliminary phenotypic and genetic characterization of the plasmids obtained in order to discuss and justify further research and whole replicons sequencing, both due to potential application in genetic engineering as cloning vectors or as vehicles for protein expression and because of the possible usefulness that the knowledge about some genes contained into it, like, for example, antibiotic or heavy metal resistance genes, may has.

3. Materials and methods

3.1 Sample characterization

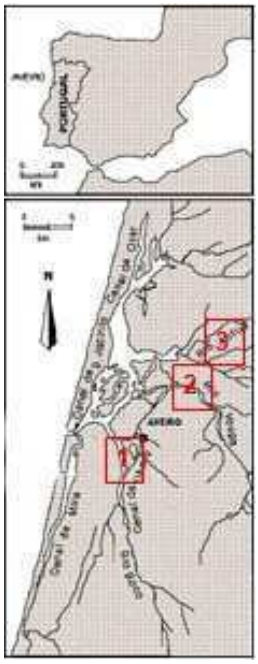


Fig 2. Sampling sites in Ria de Aveiro (*Oliveira et al., 2009).*

Samples were collected throughout 2008 and 2009 from three contrasting aquatic environments of the *Ria de Aveiro* lagoon, in the NW coast of Portugal. These sites were different as for salinity, temperature and anthropogenic pressures (run-off from agriculture [1.-*Costa Nova*-CN], port activities [2.-*Cais do Sporting*-CS] and industry [3.-*Cais do Chegado*-CC]). In each sampling site, glass plate method was employed to harvest samples from the SML and a flask submerged at a depth of 40 cm was used to gather samples from the SSW (Ramos, 2009; Oliveira et al., 2009).

On one hand, in order to determine the BHR plasmid distribution by endogenous isolation, the culturable bacterial fraction was analyzed. Right through a previous research investigation (Ramos, 2009) the bacterial strains harvested had been cultured and isolated, resulting in 402 bacterial isolates, 198 from SML and 204 from SSW.

On the other hand, a cultivation-independent approach was carried out by using *Pseudomonas putida* KT2442 and *Escherichia coli* CV601 as recipient strains to capture plasmids by conjugation. The 79 transconjugants obtained (through the selective markers ampiciline, tetracycline and mercuric chloride) were confirmed by repetitive extragenic palindromic polymerase chain reaction genomic fingerprints method in a preceding work (Oliveira et al., 2009) in which also the presence of plasmids belonging to the incompatibility groups IncN, IncQ and IncW were tested without any positive result.

3.2 BHR incompatibility group testing

In this work, the PCR amplification of nucleotide sequences of specific replicons of plasmids belonging to the incompatibility groups W, N and Q and to the incP-1 subgroups was performed in order to detect the presence of BHR plasmids in the bacterial isolates

representative from SML and SSW. Regarding the transconjugants, PCR amplifications of nucleotide sequences of specific replicons was carried out for the incP-1 incompatibility sub-groups.

All PCR reactions were carried out either on a *Bio-Rad iCycler Thermal Cycler* (Bio-Rad) or on a *Bio-Rad MyCycler Thermal Cycler* (Bio-Rad).

The detection of endogenous plasmids by PCR was performed using total DNA extracted from the bacterial isolates with the *Silica bead DNA extraction Kit* (Fermentas, K0513) as DNA template. Mixtures of the total DNA of each set of 5 bacterial isolates were prepared. After confirming the success of such DNA template dilutions, every mixture was employed as one unique sample for the screening procedure. On the other hand, IncP-1 PCR testing for the 79 transconjugants was carried out. Bacterial suspensions made up by dipping a tiny amount of fresh culture into 20 µl of sterile distilled water were employed, in this case, as DNA template.

Three different degenerative consensus primer pairs developed by Bahl and co-workers (2009) were used for the amplification of the 281 bp homologous fragments from the gene *trfA* (which encode the replication initiation protein) from plasmids belonging to different IncP-1 subgroups. Primers, as well as positive controls, are described in tables III and IV, respectively. The reaction mixture consisted in: 1x phusion HF Buffer (Promega); 2,5 mM $MgCl_2$ (Promega); 200 µM dNTP Mix (Bioron); 5% DMSO (Dimethylesulfoxide, Analitical Grade - EuroBio); 0,5 µM each primer; 1 U/µl *Taq* polymerase (Promega); 1 µl of template DNA in a 25 µl PCR reaction mixture. The PCR cycle conditions are described in table V.

As for IncQ, IncN and IncW plasmids detection, methodology and primers based on these developed by Gotz and co-workers (1996) were used. Every PCR mixture contained 1x phusion HF Buffer (Promega); 3,75 mM $MgCl_2$ (Promega); 200 µM dNTP Mix (Bioron) for IncQ_oriV and 160 µM for IncN_rep and IncW_oriT; 2 % DMSO (Dimethylesulfoxide, Analitical Grade - EuroBio, France); 0,8 µM each primer; 1 U/µl *Taq* polymerase (Promega); 1µl of template DNA up to 25 µl PCR reaction mixtures. Primers, positive controls and PCR cycle conditions are described in tables III, IV and V, respectively.

Table III. Sequence of the primers used for the amplification, target genes and expected fragment length of the polymerase chain reaction products correspondent the different incompatibility groups and subgroups tested.

BHR incompatibility group assessed	Target gene	Expected PCR products size (bp)	Forward primer		Reverse primer		Reference
			Name	Sequence(5' --> 3')	Name	Sequence (5' --> 3')	
IncP-1α, -β, -ε	*trfA*	281	trfA_fw	TTCACSTTCTACGAGMTKTGCCAGGAC	trfA_rev	GWCAGCTTGCGGTACTTCTCCCA	Bahl et al. (2009)
IncP-1γ	*trfA*	281	trfA-γ_fw	TTCACTTTTTACGAGCTTTGCAGCGAC	trfAγ_ rev	GTCAGCTCGCGGTACTTCTCCCA	Bahl et al. (2009)
IncP-1δ	*trfA*	281	trfA-δ_fw	TTCACGTTCTACGAGCTTTGCACAGAC	trfA-δ_rev	GACAGCTCGCGGTACTTTTCCCA	Bahl et al. (2009)
IncQ	*oriV*	436	oriV_1	CTCCCGTACTAACTGTCACG	oriV_2	ATCGACCGAGACAGGCCCTGC	Gotz et al. (1996)
IncN	*rep*	164	rep_1	AGTTCACCACCTACTCGCTCCG	rep_2	CAAGTTCTTCTGTTGGGATTCCG	Gotz et al. (1996)
IncW	*oriT*	317	oriT_1	TCTGCATCATTGTAGCACC	oriT_2	CCGTAGTGTTACTGTAGTGG	Gotz et al. (1996)

*primers are ordered to StabVida

Table V. PCR conditions followed for each gene target.

Target gene	Initial denaturation		Cycles	Denaturation		Annealing		Extension		Final extension	
	Temperature	Time		Temperature	Time	Temperature	Time	Temperature	Time	Temperature	Time
trfA (IncP-1α, -β, -ε)	98ºC	30 s.	35	98ºC	20 s.	67ºC	20 s.	72ºC	30 s.	72ºC	5 min
trfA (IncP-1γ)	98ºC	31 s.	35	98ºC	21 s.	67ºC	21 s.	72ºC	31 s.	72ºC	5 min
trfA (IncP-1δ)	98ºC	32 s.	35	98ºC	22 s.	67ºC	22 s.	72ºC	32 s.	72ºC	5 min
oriV (IncQ)	94ºC	5 min	35	94ºC	1 min	57ºC	1 min	72ºC	1 min	72ºC	10 min
rep (IncN)	94ºC	5 min	35	94ºC	1 min	55ºC	1 min	72ºC	1 min	72ºC	10 min
oriT (IncW)	94ºC	5 min	35	94ºC	1 min	51ºC	1 min	72ºC	1 min	72ºC	10 min

Table IV. Positive controls used in the PCR reactions for every plasmid incompatibility group or subgroup.

BHR incompatibility group assessed	***Positive controls***	
	Plasmid name	***Bacterial host***
IncP-1α	RP4	*Escherichia coli* SCS1
IncP-1β	pB10	*E.coli* JM109
IncP-1ε	pKJK5	*E.coli* CSH26
IncP-1γ	pQKH54	*E.coli* DHlOB
IncP-1δ	pEST 4011	*Achromobacter xylosoxidans*
IncQ	pJE723	*Escherichia coli* JE723
IncN	RN3	*E.coli* J53
IncW	R388	*E.coli* DH5α

3.3 PCR amplification products detection: electrophoresis and dot-blot hybridization

The PCR amplification products corresponding to IncP-1 plasmid specific sequences were separated on a 1.5% agarose (Lonza) gel in TAE (Tris-Acetate-EDTA, 5Prime) buffer at 80V during 70 minutes. Either *GeneRuler DNA Ladder Mix* (Fermentas) or *GeneRuler 100bp DNA Ladder Plus, ready-to-use* (Fermentas) were loaded in all gels as molecular weight markers. Gels were stained with ethidium bromide and banding patterns were visualized by using a *Molecular Imager FXTM system* (Bio-Rad Laboratories).

Dot-blot hybridization with digoxigenin-labeled PCR-derived probes from each IncQ, IncN and IncW templates, was employed, by using the *DIG Nucleic Acid Detection Kit* (Roche), to carry out the detection of the correspondent PCR amplification products. The compositions of all the solutions used in the procedure are described in table VI.

Detailed protocol:

- The DNA from PCR reactions was denatured by mixing 20 µl of every PCR product with 2 µl of NaOH 0.5 N and incubating at 50ºC for 5 minutes.
- 6 µl of *standard sodium citrate* (SSC) 20 X were added to every sample to neutralize them.
- The DNA transference to a previously soaked in *SSC 20X positively charged nylon transfer membrane* (Amersham Hybond –N+, GE Heathcare) was performed under vacuum at 50 mm Hg, by using a *VacuGene Pump* (VacuGene XL, Pharmacia Biotech) as vacuum blotting System.
- Dot blotted nucleic acid was UV cross-linked to the nylon membrane during 5 minutes.
- The *PersonalHyb hybridization oven* (Stratagene) with the pre-heated at the same temperature *pre-hybridation solution* was employed to carry out the pre-hybridization for 2 hours at 42ºC.
- The probes (made up through the *PCR DIG Probe Synthesis Kit*, Roche) were heated at 68ºC for 10 minutes to allow the DNA to dissociate.
- The hybridization tubes with the membranes and the *hybridization solution* (containing each one the correspondent probes) were incubated at 42ºC for 16 hours.
- The membranes were washed several times (twice with *wash solution I* for 5 minutes at room temperature, with *wash solution II* for 15 minutes at 42ºC and *maleic acid buffer* for 5 minutes at 42ºC), blocked (*blocking solution* for 30 minutes at the room temperature) and incubate with the *antibody solution* (the conjugate DIG-alkaline phosphatase in dissolved in *blocking solution*) for exactly 30 minutes at the room temperature.
- After washing (*maleic acid buffer* for 15 minutes at the room temperature) and equilibrating (*detection buffer* for 5 minutes at the room temperature), the membranes were placed on a previously prepared tray in a dark environment (without shaking).
- The chromogenic detection began when the membranes where soaked in the *detection solution*. Revealing lasted two hours and the reaction was stopped with abundant distilled water.

Table VI. Compositions of all the solutions used in the dot-blot hybridization.

pre-hybridation solution	SSC 5 X, blocking agent 1%, sarcosil 0.1%, SDS 0.02%, formamide 25%, distilled water
hybridation solution	SSC 5 X, blocking agent 1%, sarcosil 0.1%, SDS 0.02%, formamide 25%, labeled probe, distilled water
wash solution I	SSC 2 X , SDS 0,1%, distilled water
wash solution II	SSC 0.05 X , SDS 0.1%, distilled water
maleic acid buffer	0.1 M maleic acid, 0.15 M NaCl, distilled water, adjust to pH 7.5
blocking solution	blocking reagent 10% in maleic acid buffer
antibody solution	blocking reagent 9.99%, maleic acid buffer 89.99%, anti-DIG 0.02%
detection buffer	NaCl 0.1 M, MgCl2 50mM, Tris-HCl 0.1 M, distilled water, adjust to pH 9.5
detection solution	detection buffer 98%, NBT/BCIP solution (Roche) 2%

3.4 DNA sequencing of PCR products and sequence analysis

The PCR products obtained from the amplification of the nucleotide sequences from specific replicons belonging to the IncP-1incompatibility subgroups were purified with a slightly modified *JETQUICK PCR Purification Protocol* (Genomed). The DNA elution was performed by applying 20 µl of sterile distilled water at 65ºC.

The purified amplicons were sequenced through the chain termination method (Sanger et al., 1977) by the company StabVida. Then, in order to determine their closest phylogenetic relatives the *BLAST* software (Altschul et al., 1997) were used to compare the obtained sequences with known sequences. Sequences were aligned with reference taxa within the sequence databases using the *CLUSTALW2* program (Larkin et al., 2007).

Finally neighbor-joining trees based on the distance parameter were constructed using the *PAUP version4.0b10 program* (Swofford, 2003). Bootstrap values from 1000 replicates were also acquired and shown as percentages.

3.5 Plasmid DNA isolation and purification

With the purpose of further characterizing the detected BHR plasmids, the *Qiagen plasmid Mini Kit* was employed to perform the plasmid DNA purification. Extraction was

carried out following the manufacturers instructions with adaptations described below. The compositions of the solutions required in this procedure are described in table VII.

Detailed protocol:

- Starter cultures of LB medium (Miller, Merck) containing the appropriate selective markers (30 µg/ml for tetracycline and 25 µg/ml for mercury chloride) were inoculated with single colonies picked from fresh streaked plates of the transconjugants harboring the detected plasmids and incubated overnight at 28ºC with shaking of approximately 165 rpm.
- The bacterial cells from culture volumes of 25 ml were harvested by centrifugation (4500 rpm, 7 minutes, 4ºC).
- Each pellet was ressuspended with 4 ml of *Buffer P1*.
- The lysis of the cells was favored by adding 4 ml of *Buffer P2*, mixing thoroughly and incubating for exactly 5 minutes at room temperature.
- To neutralize, 4 ml of pre-chilled *Buffer P3* were added, starting immediately the incubation on ice for at least 15 minutes.
- Centrifugation (13000 rpm, 10 min, 4ºC) was carried out twice in order to eliminate unwanted cell material.
- The clear lysate (the supernatant) containing DNA was kept and split into two equal fractions to avoid subsequently over-loading the low capacity column.
- Both fractions of DNA were precipitated by adding 1 volume of room temperature isopropanol.
- Centrifugation at 13000 rpm and 4ºC for 30 minutes made unwanted metabolites such as proteins and lipopolysaccharides to be removed. At this time, one of the fractions corresponding to each sample was used to continue the protocol, while plasmid from the second one was kept at 4ºC to be purified afterwards by equilibrating again the same column and following this instructions from here on.
- When no visible liquid could be seen on the DNA pellets, they were redissolved in 100 µl dH2O and 1 ml of *Buffer QBT* was added.
- The samples were applied to the previously equilibrated with 1 ml *Buffer QBT QIAGEN-tip 20* columns, allowing them to enter the resin by gravity flow.

- The *QIAGEN-tips* were washed with 4 x 1 ml *Buffer QC*.
- The plasmid DNA was eluted by applying 1 ml *Buffer QF* (pre-heated at 65ºC to increase the yield).
- The eluted DNA was precipitated by adding 0.7 volumes of room-temperature isopropanol.
- A centrifugation at 13.000 rpm and 4ºC for 30 minutes was carried out.
- DNA pellet was washed with 1 ml room-temperature 70% ethanol and centrifugated again at 13.000 rpm and 4ºC for 10 minutes.
- The pellets obtained were redissolved in 20 µl of *TE* buffer.

The success of the plasmid purification was evaluated by loading onto 0.8% agarose (Lonza) gel in TAE (Tris-Acetate-EDTA, 5Prime) buffer at 60V for 200 min. The molecular weight marker used was *GeneRuler DNA Ladder Mix* (Fermentas). Gels were stained with ethidium bromide and banding patterns were visualized by using a *Molecular Imager FXTM system* (Bio-Rad).

Table VII. Composition and storage temperature of every solution required to perform the plasmid purification.

Buffer	***Composition***	***Storage***
Buffer P1 (resuspension buffer)	50 mM Tris·Cl, pH 8.0; 10 mM EDTA; 100 µg/ml RNase A	2–8 ℃, after addition of RNase A
Buffer P2 (lysis buffer)	200 mM NaOH, 1% SDS (w/v)	15–25 ℃
Buffer P3 (neutralization buffer)	3.0 M potassium acetate, pH 5.5	15–25 ℃ or 2–8 ℃
Buffer QBT (equilibration buffer)	750 mM NaCl; 50 mM MOPS, pH 7.0; 15% isopropanol (v/v); 0.15% Triton® X-100 (v/v)	15–25 ℃
Buffer QC (wash buffer)	1.0 M NaCl; 50 mM MOPS, pH 7.0; 15% isopropanol (v/v)	15–25 ℃
Buffer QF (elution buffer)	1.25 M NaCl; 50 mM Tris·Cl, pH 8.5; 15% isopropanol (v/v)	15–25 ℃
TE	10 mM Tris·Cl, pH 8.0; 1 mM EDTA	15–25 ℃

3.6 Restriction analysis

A preliminary genetic characterization of the captured plasmids was performed by carrying out DNA digestions with the endonuclease *NotI* (#ERO591, Fermentas). The enzyme was chosen for the restriction analysis according to the bibliography and the *in silico* analysis made by means of the *NEBcutter* software (Vincze et al., 2003). Additionally, it is known that IncP-1 plasmids are characterized by a high guanine + cytosine content. Hence, the use of an endonuclease recognizing DNA regions enriched in

these nucleotides should give back more enlightening restriction patterns which allow to better compare the plasmids with each other. The previously known pKJK5 and pB10 were used as controls.

Firstly the purified plasmid DNA was quantified using a *ND-1000 spectrophotometer* (Nanodrop). Digestions were carried out by preparing a 20 µl reaction mixture containing 0.05-0.1 mg/µl of plasmid DNA, 0.25U/ µl of *NotI*, 1 X *buffer O* (Fermentas) and sterile miliQ water and incubating for 16 hours at 37 ºC. The enzymatic reactions were stopped by freezing at -20ºC.

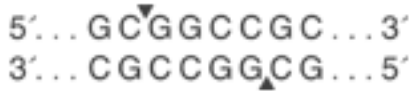


Fig 3. Nucleotide sequence recognition site of endonuclease NotI.

To analyze the resulting restriction profiles 5 µl of every restriction reaction were loaded onto 0.8% agarose TAE gels, and ran for 200 minutes at 40 V, using the *GeneRuler DNA Ladder Mix* (Fermentas) as molecular weight marker. Ethidium bromide-stained gels were seen using a *Molecular Imager FXTM system* (Bio-Rad).

3.7 Electrotransformation of *Escherichia coli* Top10 with IncP-1 plasmids

With the aim of improving the DNA quality, the purified plasmids known to encode for tetracycline resistance (from the transconjugants 1, 2, 6 and 7) were transferred to previously prepared electrocompetent well known bacterial cells (*Escherichia coli* Top10, sensible to tetracycline). The *MicroPulser™ Electroporation Apparatus* (Bio-Rad) was employed to perform a slightly modified electrotransformation procedure based on the Catalog Number 165-2100.

Detailed protocol:

- Both the electrocompetent cells and a sterile 0.1 cm electroporation cuvette (Bio-Rad) were placed on ice.

- 40 µl of the cell suspension were mixed with 2 µl of pDNA in a pre-chilled microfuge tube and incubated on ice for 1 minute.
- The mixture of cells and DNA were transferred to the bottom of the cold electroporation cuvette, which were placed in the right position in the chamber slide.
- The *Ec1* program was chosen and one fast pulse was made.
- The cuvette was immediately removed from the chamber and 450 µl of SOC medium were added without delay.
- After quickly but gently ressuspending the cells with a Pasteur pipette, the solution was cultured at 37ºC for 1h30min with shaking of 200 rpm approximately.

The success of the procedure was tested by plating in TSA medium (Tryptic Soy Broth, Merck; Rose-Bengal Chloranphenicol Agar, Merck) supplemented with 30 µg/ml of tetracycline and incubating at 37ºC.

3.8 Antibiotic resistance assays

The antibiotic resistance patterns of *Pseudomonas putida* KT2442 and Es*cherichia coli* Top10 harboring the identified incP-1 plasmids were determined by the agar disk diffusion method on Mueller Hinton media (Merck). Susceptibility testing were performed for resistance against a panel of 9 antibiotics, including representatives of the most common antibiotic resistance traits shown by the previously known IncP-1 plasmids: streptomycin (S10), amoxicillin (AML25), amoxicillin and clavulanic acid (AMC30), tetracycline (TE10), chloramphenicol (C10), trimethroprim-sulfamethoxazole (SXT25), erythromycin (E15), cephalothin (KF30) and aztreonam (ATM30). The antibiotics disks were supplied by the company Oxoid. *Escherichia coli* 25592 as well as *Pseudomonas putida* KT2442 (original host) or *Escherichia coli* Top10 (subsequent host) were used as controls.

Detailed protocol:

- To assure the use of fresh cultures (not more than 24 hours) the antibiotic assay control bacterial strain, as well as all the microorganism to test, were cultivated in LA medium (Lonka) at 30ºC (for *Pseudomonas putida*) or 37ºC (for *Escherichia coli*).

- Cellular suspensions were prepared by dipping single colonies of every fresh culture into 1 ml of $NaCl_2$ 0.9 % comparing the turbidity to the 0.5 of the McFarland scale pattern (approximately 1.5x108 CFU/ml).
- Every microorganism was spread on a Mueller Hinton media (Merck) plate, guaranteeing the whole surface covering.
- The sterile discs were placed on its correspondent positions.
- Finally the plates were incubated at 30ºC (for *Pseudomonas putida*) or 37ºC (for *Escherichia coli*) for 24h.

The diameters of inhibition zones of the Mueller-Hinton media (Merck) were afterward measured and the susceptibility/resistance breakpoints defined by the CLSI (Clinical Laboratory Standards Institute) for *Pseudomonas* or *Escherichia coli* (CLSI 2005) were considered.

3.9 $HgCl_2$ resistance assay

In order to find a connection between the presence of the captured plasmids and the levels of resistance to mercury ions, the minimal inhibitory concentrations (MIC) were determined. With that purpose, the correspondent transconjugants were plated in TSA medium (Tryptic Soy Broth, Merck; Rose-Bengal Chloranphenicol Agar, Merck) supplemented with increasing $HgCl_2$ (Sigma) concentrations: 25, 100, 200, 400 and 3200 µg/ml. Each experiment was repeated twice, except that with the highest concentration. *Pseudomonas putida* KT2442, known to be sensible to mercury ions, was used as negative control. The lowest concentration that inhibited growth compared with the control plate was defined as the MIC of the compound (Rasmussen et al., 1998).

4. Results and discussion

4.1 BHR plasmid detection and phylogenetic analysis

The presence of BHR plasmids in the *Ria de Aveiro* lagoon from endogenous and exogenous origin was examined by means of PCR amplification and subsequently checked by electrophoresis or dot-blot hybridization.

The PCR screening for endogenous BHR-plasmids was performed over representative bacterial isolates from SML and SSW environments along the lagoon. Electrophoresis were carried out for the detection of the IncP-1 amplifications, while the dot blot hybridization method was employed to test out the results of IncN, Q and W PCRs. As shown in the figs 4 and 5, no amplification of BHR-plasmid specific sequences was detected for any of the samples tested. Hence, these results suggest that no BHR plasmids are present in any of the bacterial isolates tested.

In this work, the 79 putative plasmids obtained from plasmid exogenous capture using *Pseudomonas putida* KT2442 as host were only PCR tested for IncP-1 BHR plasmid specific sequences, since in a previous investigation, no incN, Q and W replicons were detected (Oliveira et al., 2009). Fig 6 shows the PCR results using the *trfA* set of primers in 7 transconjugants. It is possible to see in the correspondent lanes the amplification of a DNA fragment of about 281bp. As for the remaining samples and PCR primers sets, no amplification was detected. The amplifications obtained for the 7 transconjugants samples most possibly correspond to the amplification of a 281bp DNA fragment of the gene *trfA* from IncP-1 α, β or ε BHR plasmids. This indicated that such IncP-1 plasmids were present in the *Ria of Aveiro* lagoon due to the specificity of the different primer pairs, respectively.

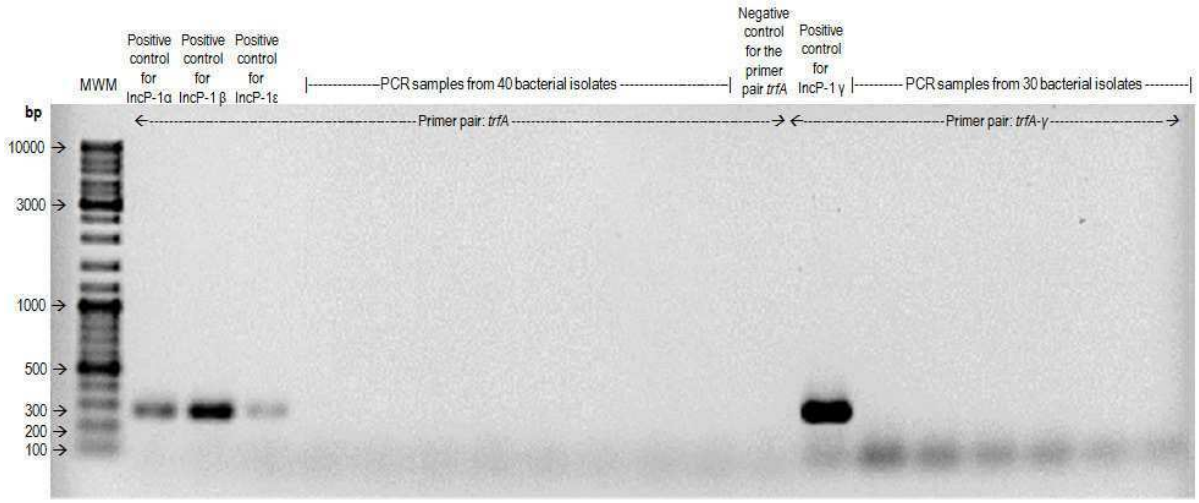
From here on, to simplify, the plasmids harbored by transconjugants 1, 2, 6-10 will be referred to in the text and tables as 1, 2, 6-10. It might be relevant to consider the divergence among these seven plasmids, both regarding the selective marker used to capture them and with respect to the origin, that is summarized in table VIII. Four of the plasmids (1, 2, 6 and 7) were captured by taking advantage of tetracycline resistance phenotypes, whereas the other three (8, 9 and 10) were seized from its resistance to mercury ions. While two (1 and 2) came from a sampling site in *Ria* where the presence of harbor facilities and urban effluents determine the selective pressures, *Cais do Sporting*,

the remaining ones (6, 7-10) emanate from a site highly impacted by the chemical industrial plants located in its vicinity, *Cais do Chegado* (Ramos, 2009). At the same time, two plasmids (9 and 10) were collected from the SSW, in contrast to the other five (1, 2, 6-8), which had their origin in the SML. That suggests wider plasmid diversity both in the SML (and in the sampling site influenced by industrial activities, *Cais do Chegado*.

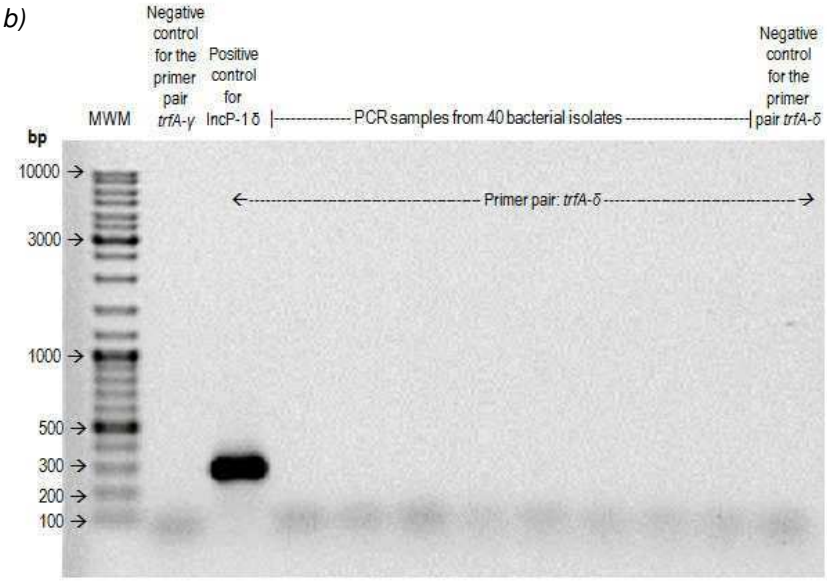
Table VIII. Features of the captured BHR plasmids from the Ria de Aveiro *lagoon.*

Number of plasmid	***Phenotype trait used for plasmid isolation***	***Origin site***	***Aquatic environment***
1	resistance to tetracycline	Cais do Sporting	SML
2	resistance to tetracycline	Cais do Sporting	SML
6	resistance to tetracycline	Cais do Chegado	SML
7	resistance to tetracycline	Cais do Chegado	SML
8	resistance to mercury ions	Cais do Chegado	SML
9	resistance to mercury ions	Cais do Chegado	SSW
10	resistance to mercury ions	Cais do Chegado	SSW

a)



b)



Figs 4a and 4b. Example of agarose gels for analysis of IncP-1 PCR amplifications. 5 µl of every amplification product were loaded onto 1.5% agarose gels and ran for 70 minutes at 80 V. Similar images were obtained in the PCR screening for IncP-1 performed over the rest of the endogenous bacterial samples tested.

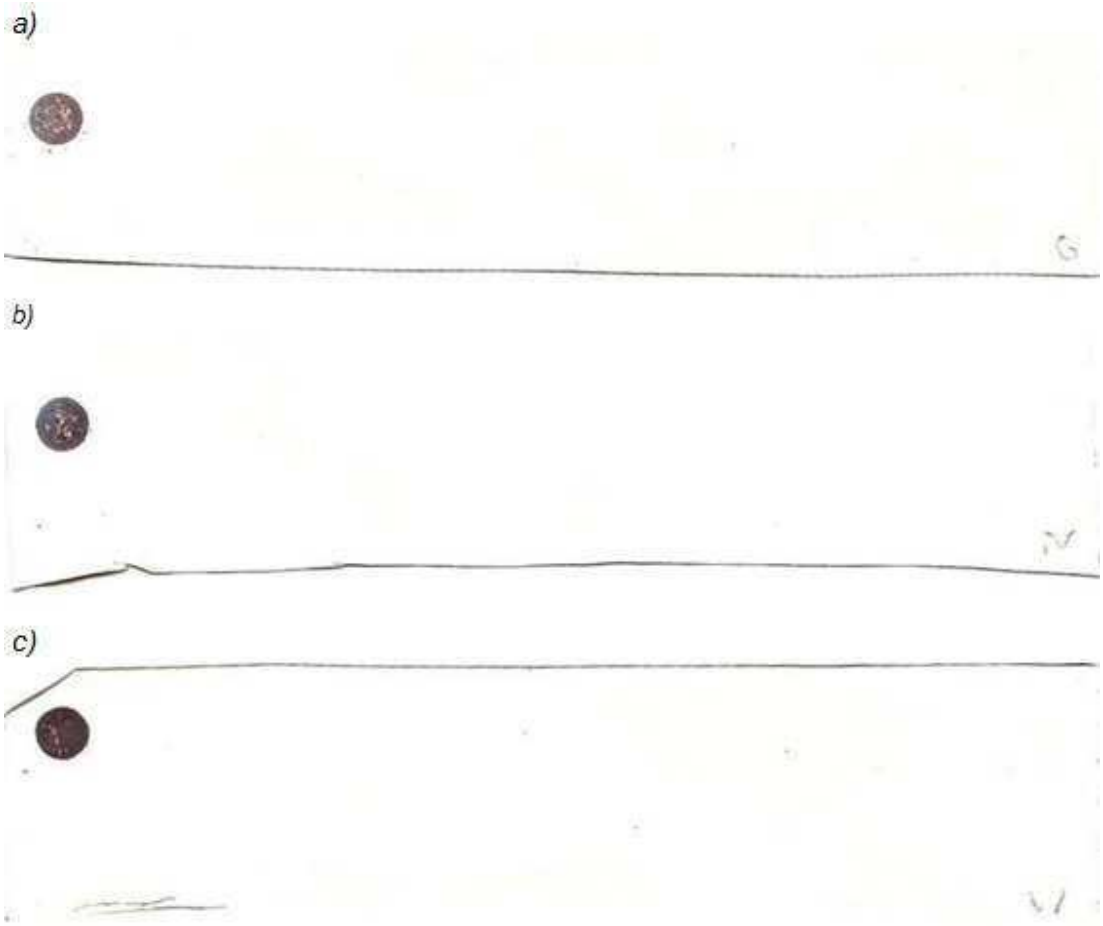


*Fig 5a, 5b and 5c. Images obtained from the dot-blot hybridization where the detection of the products from the PCR screening for IncQ (a), IncN (b) and IncW (c) plasmids performed over 40 endogenous origin bacterial isolates can be seen. The first square in each hybridization membrane correspond to the appropriate positive control for the primer pair employed in the corresponding PCR (*oriV, rep *and* oriT *for IncQ, IncN and IncW respectively) whereas the last one match up with the negative control. Analogous images resulted from the evaluation of the remaining bacterial isolates for the same incompatibility plasmid groups.*

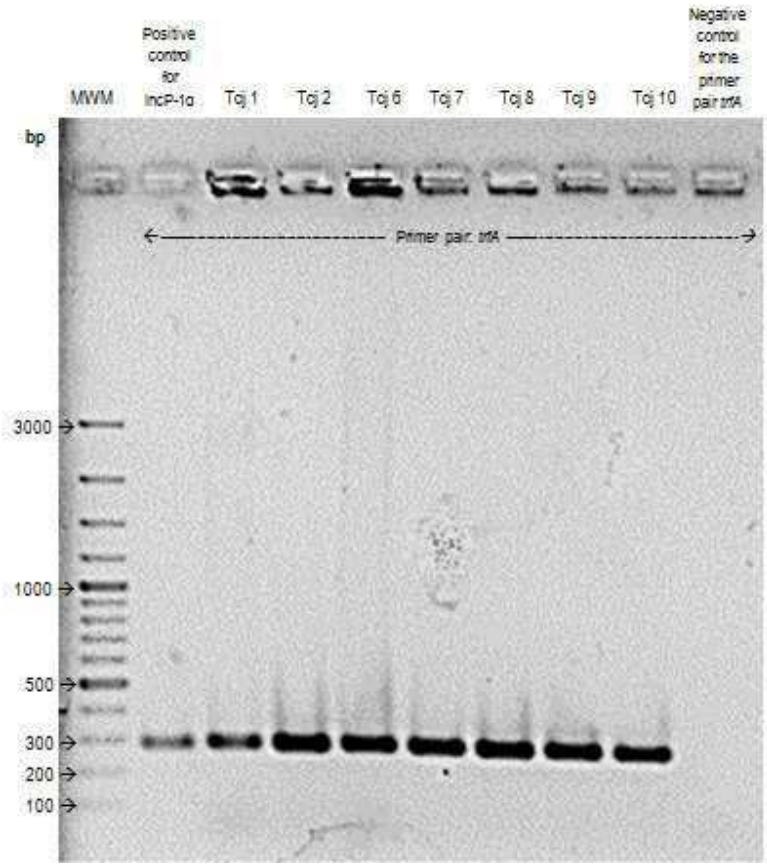


Fig 6. Image of the agarose gel where the amplification products of the PCR screening made over seven transconjugants for the IncP-1 α, β or ε BHR plasmids were detected. The samples ran for 80 minutes at 80 V in a 1.5 % agarose gel.

With the aim of identifying the IncP-1 plasmid subgroup (α, β or ε) to which each amplicon corresponded, the amplified DNA samples were purified and sequenced (Table X). Subsequently, every pair of contiguous nucleotide sequences was assembled and the resultant fragments were compared with previously known sequences by using the on-line software *BLAST* (Altschul et al., 1997). Results suggested that the plasmids 1, 6 and 7 belonged probably to the IncP-1ε subgroup whereas the 2, 8, 9 and 10 seemed to be members of the IncP-1β subgroup of plasmids. Putting together all the information known up until now (see table IX) it may be suggested that only two pairs of plasmids could be

equal: on one hand no differences were seen yet between plasmids 6 and 7 (furthermore the plasmid 1 diverges from them only in the selective pressures received at its original environment); on the other hand the plasmids 9 and 10 could be identical, differing from the remaining two plasmids (2 and 8) over the origin or/and over the phenotypic trait used for its isolation.

Table IX. Features of the captured BHR plasmids from the Ria de Aveiro *lagoon and classification into IncP-1 subgroups.*

Plasmid number	***Phenotype trait used for plasmid isolation***	***Sampling site***	***Aquatic environment***	***IncP-1 subgroup***
1	resistance to tetracycline	Cais do Sporting	SML	ε
2	resistance to tetracycline	Cais do Sporting	SML	β
6	resistance to tetracycline	Cais do Chegado	SML	ε
7	resistance to tetracycline	Cais do Chegado	SML	ε
8	resistance to mercury ions	Cais do Chegado	SML	β
9	resistance to mercury ions	Cais do Chegado	SSW	β
10	resistance to mercury ions	Cais do Chegado	SSW	β

Table X. Nucleotide sequences of the seven trfA *gene fragments amplified by PCR with the primers* trfA (5’ – 3’).

Plasmid nunber	***Nucleotide sequence of the amplified fragment***
1	TCACGTTCTACGAGATGTGCCAGGACTTGGACTGGTCGATCAACAGCCGGTACTACGCAAAGGCCGAAGAGTGCCTTAGCCGCCTGCAAGCGTC CGCGATGCAGTTTTCATCCAAGCGGATCGGCCGGCTCGAATCGCTGTCCCTGATCCGTCGCTTCCGCGTCCTGAACCGTGGCACGCGCAATTCG CGCTGCCAGGTTGAGATTGACGAAGAAATGGTTGTCCTGTTCGCCGGCGACCATTACAGCAAGTTCATTTGGGAGAAGTACCGCAAGCTGACA
2	TCACGTTCTACGAGATGTGCCAGGACTTGGACTGGTCAATCAACGGTCGGTACTACACAAGGGCCGAGGAATGCCTGACGCGGCTCCAGGCGTC GGCCATGCAGTTCTCATCCCAACGCATCGGCCGGCTCGAATCGGTGTCGCTGATCCGGCGCTTCCGCGTCCTGGATCGCGGCAAGCGCACGTCG CGCTGCCAGGTCGAGATCGACGCCGAAATCGTGGTGCTGTTCGCCGGCGACCACTACACGAAATTCGTGTGGGAGAAGTACCGCAAGCTGACA
6	TTCACGTTCTACGAGCTGTGCCAGGACTTGGACTGGTCGATCAACAGCCGGTACTACGCAAAGGCCGAAGAGTGCCTTAGCCGCCTGCAAGCGTC CGCGATGCAGTTTTCATCCAAGCGGATCGGCCGGCTCGAATCGCTGTCCCTGATCCGTCGCTTCCGCGTCCTGAACCGTGGCACGCGCAATTCG CGCTGCCAGGTTGAGATTGACGAAGAAATGGTTGTCCTGTTCGCCGGCGACCATTACAGCAAGTTCATTTGGGAGAAGTACCGCAAGCTGAC
7	TCACGTTCTACGAGCTGTGCCAGGACTTGGACTGGTCGATCAACAGCCGGTACTACGCAAAGGCCGAAGAGTGCCTTAGCCGCCTGCAAGCGTC CGCGATGCAGTTTTCATCCAAGCGGATCGGCCGGCTCGAATCGCTGTCCCTGATCCGTCGCTTCCGCGTCCTGAACCGTGGCACGCGCAATTCG CGCTGCCAGGTTGAGATTGACGAAGAAATGGTTGTCCTGTTCGCCGGCGACCATTACAGCAAGTTCATTTGGGAGAAGTACCGCAAGCTGACA
8	TTCACGTTCTACGAGATGTGCCAGGACTTGGACTGGTCAATCAACGGTCGGTACTACACAAGGGCCGAGGAATGCCTGACGCGGCTCCAGGCGTC GGCCATGCAGTTCTCATCCCAACGCATCGGCCGGCTCGAATCGGTGTCGCTGATCCGGCGCTTCCGCGTCCTGGATCGCGGCAAGCGCACGTCG CGCTGCCAGGTCGAGATCGACGCCGAAATCGTGGTGCTGTTCGCCGGCGACCACTACACGAAATTCGTGTGGGAGAAGTACCGCAGCTGACA
9	TTCACGTTCTACGAGATGTGCCAGGACTTGGACTGGTCAATCAACGGTCGGTACTACACAAGGGCCGAGGAATGCCTGACGCGGCTCCAGGCGTC GGCCATGCAGTTCTCATCCCAACGCATCGGCCGGCTCGAATCGGTGTCGCTGATCCGGCGCTTCCGCGTCCTGGATCGCGGCAAGCGCACGTCG CGCTGCCAGGTCGAGATCGACGCCGAAATCGTGGTGCTGTTCGCCGGCGACCACTACACGAAATTCGTGTGGGAGAAGTACCGCAGCTGACA
10	TCACGTTCTACGAGATGTGCCAGGACTTGGACTGGTCAATCAACGGTCGGTACTACACAAGGGCCGAGGAATGCCTGACGCGGCTCCAGGCGTC GGCCATGCAGTTCTCATCCCAACGCATCGGCCGGCTCGAATCGGTGTCGCTGATCCGGCGCTTCCGCGTCCTGGATCGCGGCAAGCGCACGTCG CGCTGCCAGGTCGAGATCGACGCCGAAATCGTGGTGCTGTTCGCCGGCGACCACTACACGAAATTCGTGTGGGAGAAGTACCGCAAGCTGACA

To further analyze the sequences, multiple alignments of these seven nucleotide sequences were performed (Fig 7). It can be noticed that the sequences from plasmids placed in the same IncP-1 subgroup are almost identical (with parwise alignment scores of 99-100), whereas sequences from different subgroups are not so similar (with parwise alignment scores of 84-85). That supports the above mentioned relationships between the seven captured plasmids.

SeqA	Name	Len(nt)	SeqB	Name	Len(nt)	Score
1	Tcj1	281	2	Tcj2	281	85
1	Tcj1	281	3	Tcj6	281	99
1	Tcj1	281	4	Tcj7	281	99
1	Tcj1	281	5	Tcj8	281	85
1	Tcj1	281	6	Tcj9	281	85
1	Tcj1	281	7	Tcj10	281	85
2	Tcj2	281	3	Tcj6	281	84
2	Tcj2	281	4	Tcj7	281	85
2	Tcj2	281	5	Tcj8	281	99
2	Tcj2	281	6	Tcj9	281	99
2	Tcj2	281	7	Tcj10	281	100
3	Tcj6	281	4	Tcj7	281	99
3	Tcj6	281	5	Tcj8	281	84
3	Tcj6	281	6	Tcj9	281	84
3	Tcj6	281	7	Tcj10	281	84
4	Tcj7	281	5	Tcj8	281	84
4	Tcj7	281	6	Tcj9	281	84
4	Tcj7	281	7	Tcj10	281	85
5	Tcj8	281	6	Tcj9	281	100
5	Tcj8	281	7	Tcj10	281	99
6	Tcj9	281	7	Tcj10	281	99

Fig 7. Score table of the multiple sequence alignments of the amplified trfA *gene fragments from captured plasmids from* Ria de Aveiro *lagoon (Tcj 1, 2, 6-10) performed by* ClustalW2.

Also, alignments were carried out together with corresponding nucleotide sequences from 20 other incP-1 plasmids that, so far, represent the full known diversity within this 281bp-region (Balh et al., 2009). Alignments were performed by the *CLUSTALW2* program.

Neighbor-joining trees based on the distance parameter were constructed, from a multiple alignment performed with the *ClustalX* program (Larkin et al., 2007), using the *PAUP4.0b program* (Swofford, 2003). Bootstrap values from 1000 replicates were also acquired and shown as percentages (see fig 8).

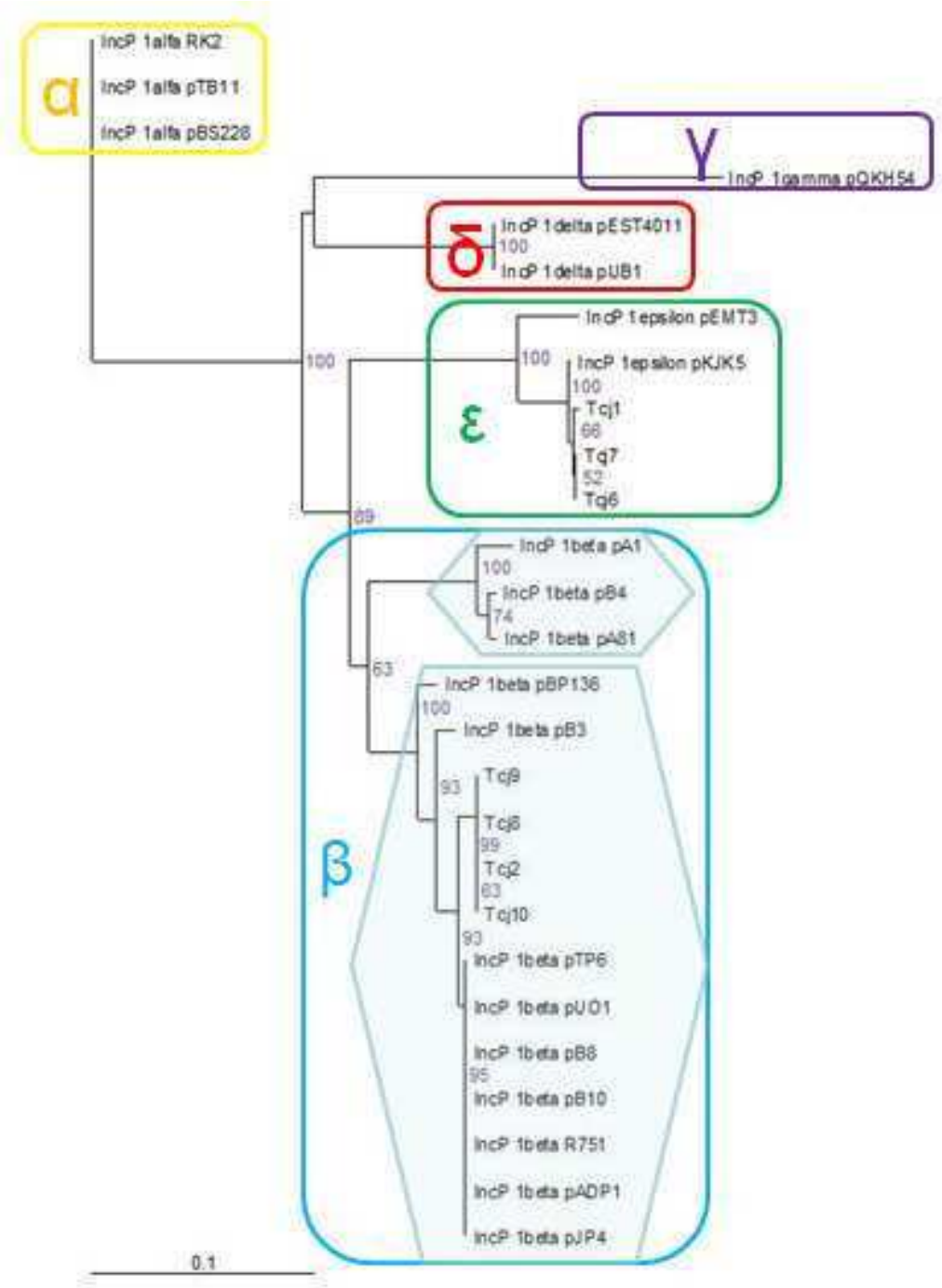


Fig 8. Phylogeny of the amplified trfA *gene fragments from captured plasmids from* Ria de Aveiro *lagoon (Tcj 1, 2, 6-10) in relation to previously described IncP-1 plasmids, based on a multiple alignment (ClustalX) of the nucleotide sequences. PAUP4.0b program was used to construct the neighbor-joining tree. Bootstrap values based on 1000 replicates are shown as percentages. The distances between the fragments are indicated by the substitutions/site bar.*

The phylogenetic analysis made was compared with the one presented by Bahl et al. (2009) since the same parameters were used. Both unrooted trees are very similar. Although a few differences can be seen in some phylogenetic associations with low bootstrap values (and therefore without a considerable significance), the greater part of the members of the IncP-1 plasmids maintain the same phylogenetic distances.

By assesing the phylogenetic tree (Fig 8) it was possible to confirm that the *trfA* gene amplificated fragments of the plasmids 2, 8, 9 and 10 belong to the IncP-1β subgroup. Additionally, the *trfA* gene fragments nucleotide sequences 8 and 9 showed to be closer philogenetycally than with the other 2 and 10. That does not totally agree with the suppositions made before, since the plasmids 9 and 10, which had in common the same original environment and were captured by means of the same phenotype, seemed to havea high chance that they could be the same plasmid. Therefore, a feasible divergence beetween plasmids with the same origin is suggested. Plasmids 8 and 9 differ only in the aquatic environment where they were captured (SML vs. SSW), since they coexisted with the same selective pressures (industries effluents in *Cais do Chegado*) and both are resistant to mercury ions. The plasmid 2 is the only one among those four which was captured by taking advantage of to its tetracycline resistance and in *Cais do Sporting* (harbor and urban effluents).Furthermore, these 4 nucleotide sequences most likely form a new cluster, hence revealing to be more different from any other incP-1β gene fragment sequences known up till now.

Regarding the plasmids 1, 6 and 7 (all of them captured from the SML taking advantage of its tetracycline resistance), the analysis of the *trfA* gene fragments corroborate that they belong to the IncP-1ε subgroup and display a higher similarity between plasmids 6 and 7 (which come from *Cais do Chegado*) than with the other captured IncP-1ε plasmid, plasmid 1 (captured from *Cais do Sporting*). That suggests a slightly different evolution related to the selective pressure occurring in the samples origin. Again, the 3 nucleotide sequences emerge like a new cluster, appearing to diverge both from the closest known IncP-1ε plasmid, pKJK5, and from the partially described pEMT3.

4.2 Plasmid characterization

With the purpose of further characterizing the captured plasmids a preliminary phenotypic and genotypic analysis was carried out. To perform this work, the two closest relatives to both clusters of plasmids based on the phylogenetic analysis described above, pB10 and pKJK5, were chosen as archetypes for IncP-1β and ε subgroups respectively.

Initially, the seven plasmids were purified from *Pseudomonas putida* KT2442, the recipient strain used in the exogenous plasmid capture experiments (Oliveira et al., 2009). The first key fact to take into account to carry out the plasmid isolation is that IncP-1 plasmids are present in the host cell in a very low copy number. As a consequence, large culture volumes were required to yield significant amounts of plasmid DNA. In addition, this family of BHR plasmids is characterized by its enormous size: all the IncP-1 plasmids know until now have more than 45 kb. Because of that its electrophoretic mobility in comparison with genomic DNA is expected to be very similar, complicating the plasmid DNA purity analysis. Anyhow, the success of the plasmid purification can be noticed in the fig 9. A subtle difference in the position of both kinds of DNA can be appreciated. Additionally, a second DNA conformation in the plasmid 10 is detected, although may be considered an artifact from the plasmid purification procedure.

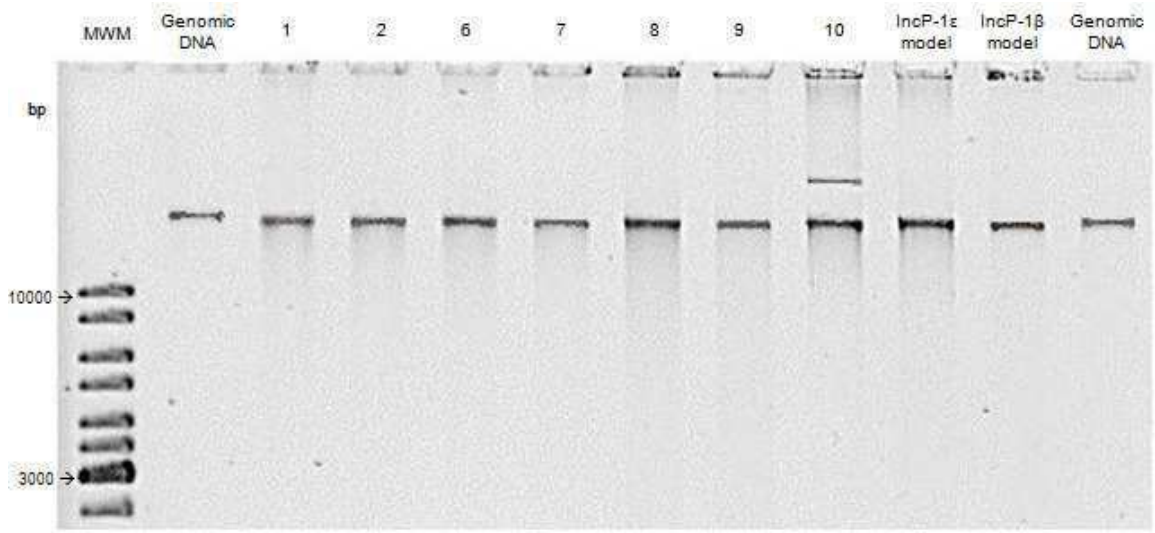


Fig 9. Illustration of the 0,8% agarose gel where every purified plasmid DNA as well as the genomic DNA extracted from Pseudomonas putida KT2442 *(the bacterial host in every cases) ran for 200 minutes at 60 V.*

Restriction fragment analyses were carried out to compare genetically the isolated plasmid DNAs. Different band profiles were obtained through the digestion of each one of the seven purified plasmids as well as the two models with the endonuclease *NotI*. The results (figs 10 and 11) allow affirming that six plasmids (1, 2, 6, 7, 8/9, 10) isolated from the *Ria de Aveiro* lagoon are different between each other and between the two fully characterized archetypes used.

In the first place, if both images are compared, the restriction patterns from both IncP-1β and IncP-1ε plasmid subgroups can be clearly distinguished, which confirm again the location of the captured plasmids into the two divergent IncP-1 subgroups that had already been proved.

Analyzing deeply the figure 10 it can be noticed that the restriction profiles of three of the captured plasmids (2, 10 and 8 or 9) are visibly different between each other and from its closest relative, whereas the plasmids 8 and 9 seem similar by looking at that image. That agrees with the phylogenetic analysis, which indicated a higher similarity between these two plasmids (8 and 9), despite the two analysis strategies consider different characteristics of these DNA molecules: the exact nucleotide sequence of a gene fragment and the position of the recognition sites of the endonuclease *NotI* in the whole unknown nucleotide sequence. In addition, plasmids 8 and 9 seems to be the closest to the previously described IncP-1β model pB10 attending to the restriction profiles. The three plasmids, coming from different environments (the SSW and the SML of the estuarine waters of *Cais do Chegado* in the *Ria de Aveiro* lagoon in Portugal and activated sludge from a municipal wastewater treatment plant in Germany, respectively) are resistant to mercury ions (Schlüter et al., 2003),

By examining the restriction profiles of the plasmids 1, 6 and 7, it can be assured that they are undoubtedly different between each other and from the unique fully sequenced plasmid of the IncP-1ε subgroup. The four plasmids carry resistance to tetracycline, while the estuarine origin of the captured plasmids is really different from the non-aquatic origin of the model, since the plasmid pKJK5 was isolated from a mixture of soil (from an agricultural field in Denmark) and manure (collected from a pig farm in the vicinity) (Bahl et al., 2007). Nonetheless, despite the similar or equal origin of the plasmids 1, 6 and 7 (the SML from *Cais do Sporting* for the first one and from *Cais do Chegado* for

the others), the genetic structure of the three IncP-1ε captured plasmids are clearly different.

Hence, it is demonstrated that there is an appreciable diversity of IncP-1 plasmids in the estuarine environment studied, even among plasmids captured from the same environment (whose evolution have been subjected to the same selective pressures).

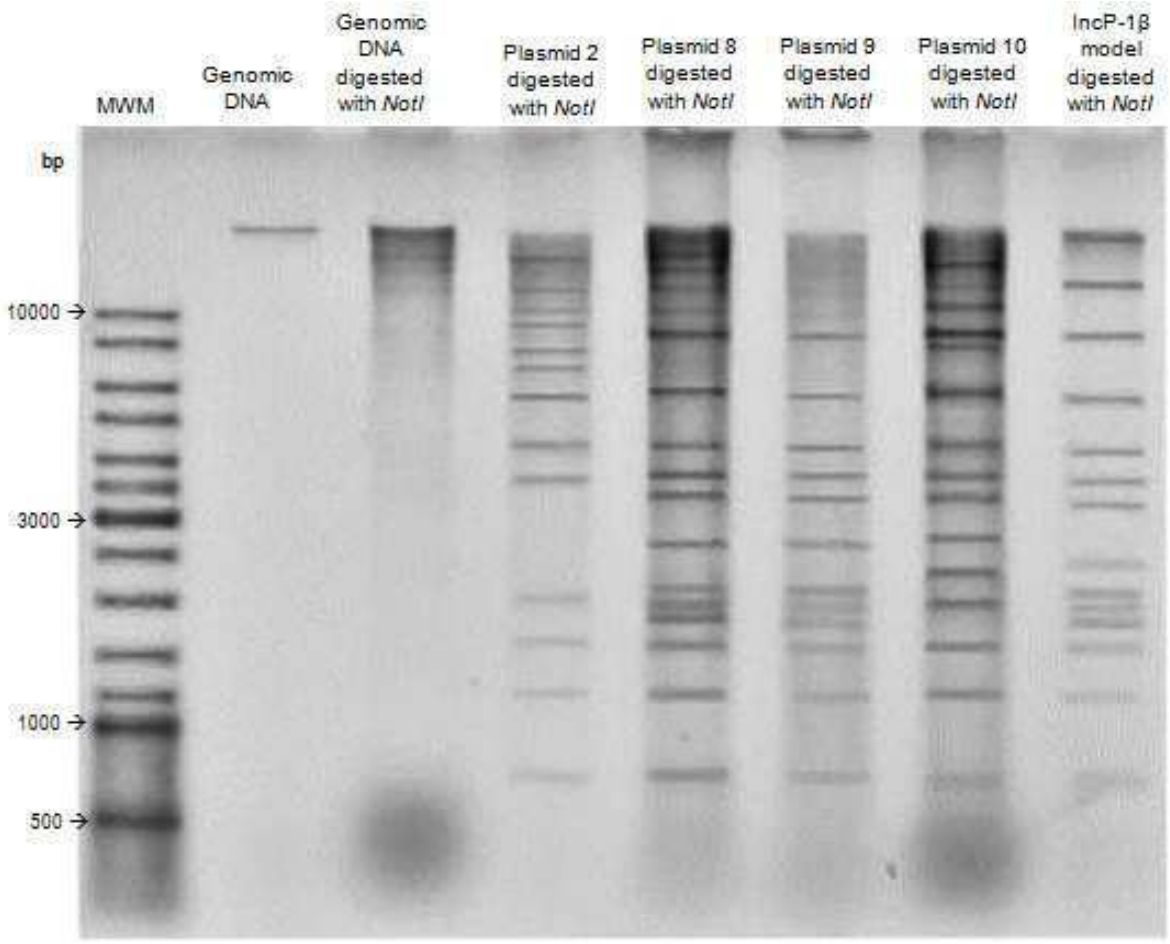


Fig 10. Illustration of the 0.8% agarose gel showing the restriction fragment profiles of the plasmids 2, 8, 9 and 10 (associated to the IncP-1β subgroup) and the known plasmid pB10 used as archetype of the IncP-1β subgroup, digested with NotI. *The DNA fragments were electrophoretically separated in a 0.8 % agarose gel by running for 200 minutes at 40 V. Genomic DNA from the bacterial host without any plasmid (*Pseudomonas putida *KT2442) with and without digestion with the same endonuclease were employed as control.*

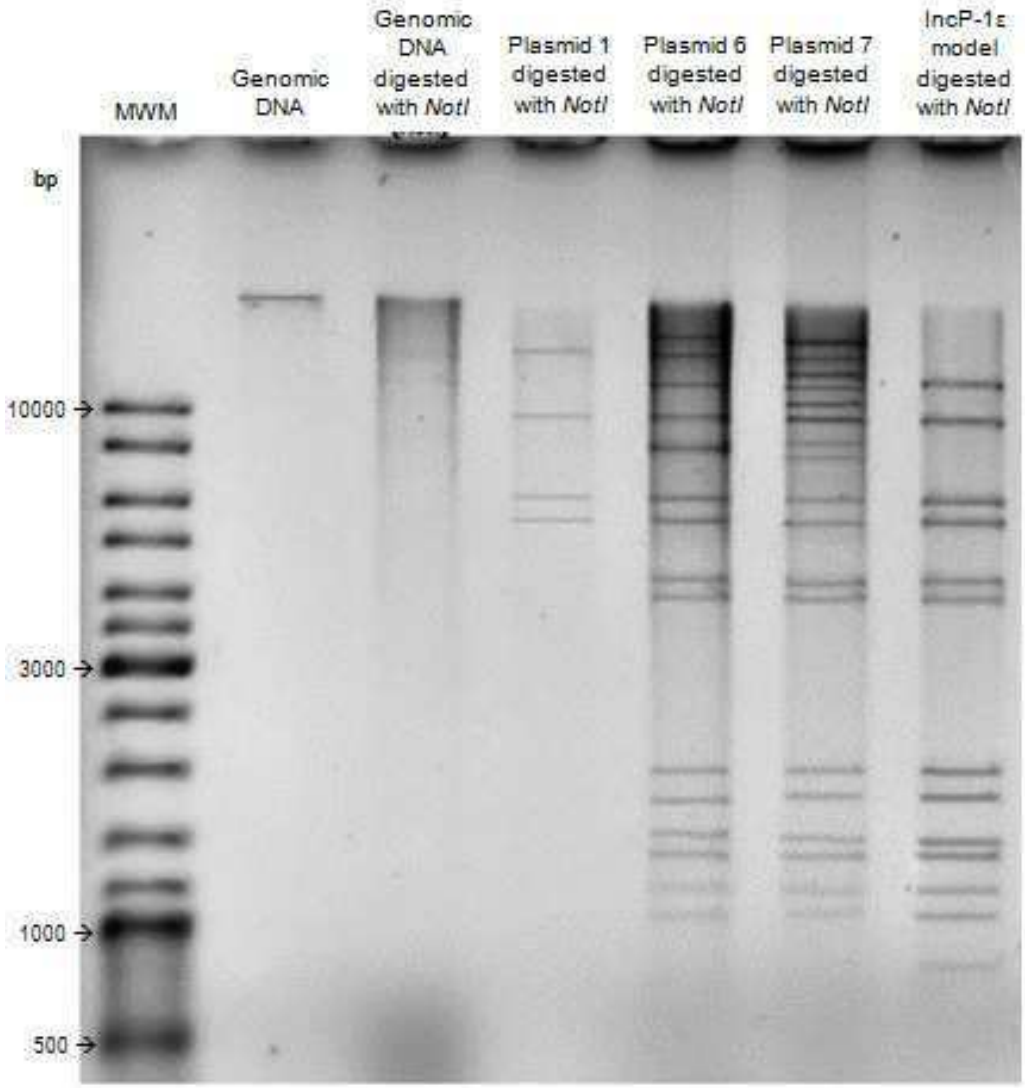


Fig 11. Illustration of the 0.8 % agarose gel where the restriction fragment profiles of the plasmids 1, 6 and 7 (related to IncP-1ε) and the known plasmid pKJK5 used as archetype of the IncP-1ε subgroup digested with NotI *were seen after running for 200 minutes at 40 V. Genomic DNA from the bacterial host without any plasmid* (Pseudomonas putida KT2442) *with and without digestion with the same endonuclease were used as control.*

It can also be perceived when seeing these gels that the plasmid purification of the seven plasmids harbored by *Pseudomonas putida* KT2442 was not perfect, since an evident difference in the DNA quality shown in the restriction profiles of the seven captured plasmids in comparison with the restriction patterns obtained from the two models can be seen. That could be due to some degradation caused by the purification procedure, and/or to a slightly contamination of the plasmid DNA with genomic DNA from the bacterial host (*P.putida).* The fact that the plasmid purification of the models (harbored by two different *Escherichia coli* strains) give such ideally purified results leads to the performance of the transference of four of the plasmids purified from *P.Putida* KT2442 (the four that were captured from its tetracycline resistance) to *E.coli* Top10 by electroporation, with the aim of improving the yield and the quality of the plasmid DNA.

Additionally, a phenotypic plasmid characterization was carried out. Firstly, some antibiotic resistance assays were done with the seven plasmids harbored by *Pseudomonas putida* KT2442, confirming that the plasmids 1, 2, 6 and 7 encode resistance to tetracycline, since the bacterial host without plasmid is susceptible to this antibiotic. It was also demonstrated that the plasmids 8, 9 and 10 do not encode resistance to this antibiotic. However, further resistance tests were hindered since the host showed resistance to the remaining eight antibiotics to be tested. The results of these assays are shown in fig 12 and explained in Table XI.

Despite that at a first glance these results appear strange (because of the antibiotic resistance trait in the bacterial host without plasmid that disappears with the presence of a plasmid), the length of the inhibition hales gives low meaning to this result. The expression of any phenotypic trait encoded by the bacterial chromosome, like the antibiotic resistances, might be decreased due to the presence of a plasmid burden. That happens with chloranphenicol and aztreonam resistance when plasmids 2, 6-10 are present, with amoxicillin/clavulanic acid resistance because of the occurring of plasmids 1, 6-10 and with streptomycin in the case of the plasmid 7.

It also should be noticed that both models are also resistant to tetracycline. That results especially interesting with respect to the IncP-1β subgroup, since three of the captured plasmids (8-10) are not resistant to this antibiotic.

Table XI. Results obtained from the antibiotic resistance assays carried out with the seven captured plasmids harbored by Pseudomonas putida *KT2442.*

	Pseudomonas putida KT2442							
Antibiotic resistance/susceptibility	***without any plasmid (Negative control)***	***with plasmid 1***	***with plasmid 6***	***with plasmid 7***	***with plasmid 2***	***with plasmid 8***	***with plasmid 9***	***with plasmid 10***
tetracycline (TE10)	susceptible	resistant	resistant	resistant	resistant	susceptible	susceptible	susceptible
trimethroprim-sulfamethoxazole (SXT25)	resistant	resistant	resistant	resistant	resistant	resistant	resistant	resistant
chloramphenicol (C10)	resistant	resistant	There was an inhibition hale of 8 mm					
streptomycin (S10)	resistant	resistant	resistant	slight resistance	resistant	resistant	resistant	resistant
amoxycilin (AML25)	resistant	resistant	resistant	resistant	resistant	resistant	resistant	resistant
amoxicillin and clavulanic acid (AMC30)	resistant	There was an inhibition hale of 10 mm			resistant	There was an inhibition hale of 10 mm		
erythromycin (E15)	resistant	resistant	resistant	resistant	resistant	resistant	resistant	resistant
cephalothin (KF30)	resistant	resistant	resistant	resistant	resistant	resistant	resistant	resistant
aztreonam (ATM30)	slight resistance	slight resistance	Also resistant, but with a larger inhibition hale					

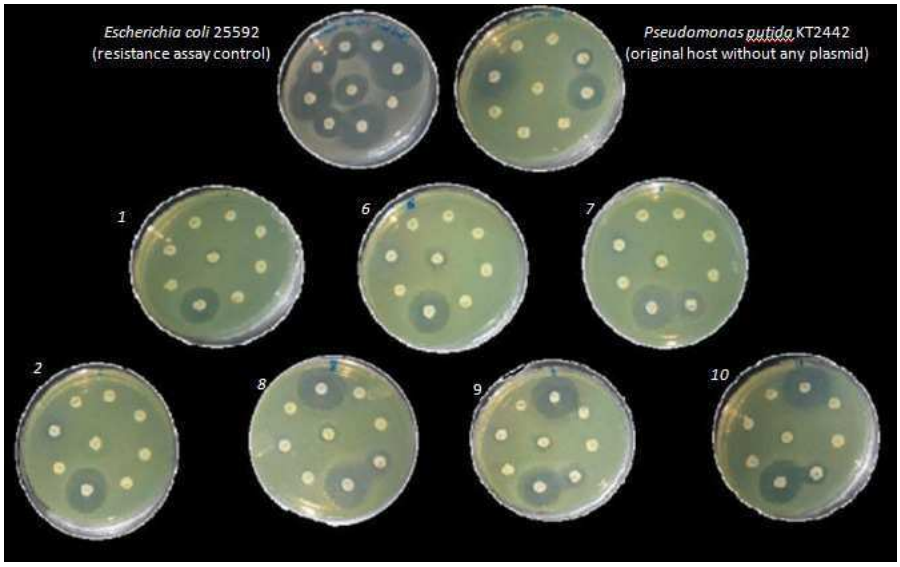


Fig 12. Image of the Mueller-Hinton medium plates obtained from the antibiotic resistance assay made with the seven plasmids harbored by Pseudomonas putida KT2442.

In order to clarify the results obtained from the first antibiotic resistance assay, a second one was performed to test the same plasmids harbored by a different host, the previously referred *E.coli* Top10. Nonetheless, only one more trait was discovered: the plasmid 2 encodes for resistance to trimethoprim-sulfamethoxazole. The results of the assays are shown in fig 13 and explained in Table XII.

Fig 13. Image of the Mueller-Hinton medium plates obtained from the antibiotic resistance assay made with the four plasmids harbored by Escherichia coli *Top10*.

Table XII. Results obtained from the antibiotic resistance assays carried out with the four captured plasmids harbored by Escherichia coli Top10*.*

	Escherichia coli Top10				
Antibiotic resistance/susceptibility	***without any plasmid (Negative control)***	***with plasmid 1***	***with plasmid 6***	***with plasmid 7***	***with plasmid 2***
tetracycline (TE10)	susceptible	resistant	resistant	resistant	resistant
trimethroprim-sulfamethoxazole (SXT25)	susceptible	susceptible	susceptible	susceptible	resistant
chloramphenicol (C10)	susceptible	susceptible	susceptible	susceptible	susceptible
streptomycin (S10)	resistant	resistant	resistant	resistant	resistant
amoxycilin (AML25)	susceptible	susceptible	susceptible	susceptible	susceptible
amoxicillin and clavulanic acid (AMC30)	susceptible	susceptible	susceptible	susceptible	susceptible
erythromycin (E15)	resistant	resistant	resistant	resistant	resistant
cephalothin (KF30)	susceptible	susceptible	susceptible	susceptible	susceptible
aztreonam (ATM30)	susceptible	susceptible	susceptible	susceptible	susceptible

Additionally, a mercury resistance assay was also performed (results are shown in Table XIII). According to Rasmussen (1998), the lowest metal concentration that inhibits growth compared with the control plate is defined as the MIC of the compound. Then despite the bacterial strains that carry plasmids 8, 9 and 10 are sensible to a concentration similar or higher than 400 of $HgCl_2$ µg/ml it can be affirmed that plasmids 8, 9 and 10 encode resistance to mercury ions. The other plasmids don't encode this resistance.

All the captured plasmids which showed resistance to mercury ions belong to the IncP-1β subgroup. The model pB10, representing this subgroup in this research, was also described to be resistant to mercury ions. Unlike, the remaining member or this subgroup within the captured plasmids, the plasmid 2, is sensible to this heavy metal presence.

Table XIII. Results obtained from the mercury resistance assay carried out with the seven captured plasmids harbored by Pseudomonas putida *KT2442.*

	Pseudomonas putida *KT2442*							
HgCl2 work concentration (µg/ml)	***without any plasmid (Negative control)***	***with plasmid 1***	***with plasmid 2***	***with plasmid 6***	***with plasmid 7***	***with plasmid 8***	***with plasmid 9***	***with plasmid 10***
25	no growth	no growth	no growth	no growth	no growth	high growth	high growth	high growth
100	no growth	no growth	no growth	no growth	no growth	high growth	high growth	high growth
200	no growth	no growth	no growth	no growth	no growth	low growth	low growth	low growth
400	no growth	no growth	no growth	no growth	no growth	no growth	no growth	no growth
3200	no growth	no growth	no growth	no growth	no growth	no growth	no growth	no growth

no growth = susceptible; high growth = resistant; low growth = intermediate (MIC)

5. General discussion and conclusions

Previous ecological studies, by means of screening of environmental DNA, on the incidence of BHR plasmids in the estuarine environment *Ria de Aveiro* lagoon suggested that there may be an interesting presence of BHR plasmids in this special niche. Therefore, two approaches were used in preceding investigations to capture these promiscuous plasmids: endogenous and exogenous isolations, in which also the presence of plasmids belonging to the incompatibility groups IncN, IncQ and IncW were tested without any positive result. In this work, the occurrence of IncP plasmids over the bacterial isolates representing contrasting environments from the lagoon and the incidence of IncP, IncQ, IncN and IncW plasmids over the transconjugants obtained from the same sample sites were tested by PCR. That strategy is not absolutely reliable, but has limitations which lead to a biased analysis. The fact that the primers are constructed only from previously known sequences is the main handicap since that disables the detection of replicons without these specific nucleotide series. That way, the inability to find replicons where the evolution has pushed any significant change in the target sequences is assumed.

No BHR plasmid presence appeared among endogenous bacteria by means of the PCR screening. Due to the restrictions of the screening method this doesn't assure the absence of this sort of plasmids among the assayed samples, but just suggests that there are not BHR plasmids containing the specific nucleotide sequences employed as targets for every incompatibility group tested amongst the cultures related to the endogenous isolation approach. The same can be explained regarding to IncQ, IncN and IncW plasmids occurring in the samples coming from the exogenous isolation.

In contrast, seven transconjugants containing IncP-1 plasmids came up as a result of exogenous isolation with *Pseudomonas putida* KT2442 as recipient strain, which proved the presence of IncP-1 plasmids in the *Ria de Aveiro* lagoon. The importance of this comes from that it is the first time that IncP-1 plasmids are isolated from an estuarine environment. In addition, that strongly suggests that the special conditions occurring in this environment stimulated the flow of MGE, allowing bacterial evolution.

The divergence among the seven captured plasmids, both with regard to the phenotype trait employed for its selection (tetracycline resistance in the case of the plasmids 1, 2, 6 and 7; resistance to mercury ions for 8, 9 and 10) and in connection with

the contrasting environments along the lagoon they were collected from (SML for 1, 2, 6-8 vs. SSW for 9 and 10; *Cais do Sporting* for 1 and 2 vs. *Cais do Chegado* for 6-10), should be considered. A wider plasmid diversity might be assumed, therefore, both in the SML (where the particular surrounding circumstances are known to favor the HGT) and in the sample site *Cais do Chegado* (where the high pressure made by chemical industrial effluents may lead the evolution).

The seven 281 bp amplicons were purified, sequenced and analyzed to be phylogenetically affiliated into its correspondent IncP-1 subgroups. Four plasmids (2, 8, 9 and 10), coming from the different locations above explained, three of them conferring mercury resistance and one tetracycline resistance (2), were confirmed to belong to the IncP-1β subgroup. And the other three plasmids (1, 6 and 7), captured from the SML of two estuarine environments from its tetracycline resistance, were determined to belong to the IncP-1ε subgroup.

Regarding the IncP-1β subgroup, a sub-division into two different phylogenetic branches had previously been suggested (Schlüter et al., 2003) by means of multiple alignments of several sequences. That divergence was also displayed in the phylogenetic tree presented here, which in addition to its general similarity with the neighbor-joining tree presented in the work of Bahl and colleagues (2009) adds soundness to the obtained phylogeny. This sub-division within this subgroup was not observed among the plasmids obtained from the lagoon, but all the four IncP-1β captured plasmids group together in a cluster contained in the same branch as the fully described archetype pB10, one of the closest relatives. The plasmid pB10 was isolated from a different environment (activated sludge from municipal wastewater treatment plant in Germany) and was reported (Schlüter et al., 2003) to be resistant to tetracycline (like the IncP-1β captured plasmid 2) and to mercury ions (like 8, 9 and 10).

No more than two IncP-1ε plasmids have been described up till now and can be used to analyze this subgroup. Barely a fragment of one of them (pEMT3) is known, but the three IncP-1ε captured plasmids are evolutionary closer to the plasmid that has been completely sequenced recently (pKJK5). However, the three IncP-1ε captured plasmids group also together in a cluster. In a really different environment (a mixture of soil and manure in Denmark), the model plasmid pKJK5 was captured thanks to the same phenotypical trait as the three IncP-1ε captured plasmids: the resistance to tetracycline

(Bahl et al., 2007). Plasmids belonging to this subgroup have also been isolated exogenously from piggery manure (Binh et al., 2008), showing *trfA* homologs of 99-100% identity to pKJK5, this sturdily indicates the presence of plasmids belonging to the IncP-1ε subgroup, which clearly diverge from the other IncP-1 subgroups, and increases the importance of further research on these plasmid subgroup.

The originality of the discovery of such two novel clusters supports the significance of this work. Therefore, in order to confirm whether both sets of plasmids really differed from all the already characterized plasmids, and to check if the captured plasmid were different from each other, a genetic and phenotypic comparison was carried out. With that purpose the evolutionary closest previously described plasmid models above explained (pB10 and pKJK5 for IncP-1β and IncP-1ε subgroups respectively) were employed. A summary of the results of this preliminary phenotypic plasmid characterization of each one of the seven plasmids in comparison to the known characteristics of the models, as well as the origins of all of them (mentioning the selective marker used in its isolation) is shown in Table XIV.

The four plasmids confirmed to belong to the IncP-1β plasmid subgroup (2, 8, 9 and 10) are clearly different from the model pB10, both attending to their phenotypic traits (the captured plasmids are resistant either to tetracycline or to mercury ions, while the plasmid pB10 is resistant to both) and to the restriction profiles (pB10 has a genetic structure clearly divergent from the rest). At the same time one of them (2), the only one which was isolated from *Cais do Sporting*, is which most differs from the remaining three, both looking at the restriction profiles and considering the phenotypic characterization (the plasmid 2 is the unique one among the members of this cluster which carries resistance to tetracycline and to trimethroprim-sulfamethoxazole). Maybe the different sampling site (and therefore the different selective pressures suffered) is related to the difference in phenotype. Although no differences in phenotypic resistance profiles were seen between the plasmids 8, 9 and 10 (all the three are resistant to mercury ions), the restriction profiles suggests that there are differences in their genetic structures. Nevertheless, while these differences are not very clear between the plasmids 8 and 9 (the closest in the phylogenetic tree), this divergence is obvious with the plasmid 10, which coming exactly from the same sampling site as the plasmid 9, differ clearly from it.

Despite the phenotypic characterization of the captured IncP-1ε plasmids (1, 6 and 7) was similar, the manifest difference of its genetic structures from the unique previously fully described plasmid pKJK5 involves a great significance, increased due to the lack of knowledge about this IncP-1 plasmid subgroup. Moreover, the restriction profiles proved also the divergence among the three.

So, overall, the detection and preliminary characterization of IncP-1 plasmids in the *Ria de Aveiro* lagoon suggested that this environment encloses high IncP-1 plasmid diversity, which indicates that these promiscuous plasmids evolved differently from the plasmids known until now, possibly due to the special conditions occurring in that niche. The fact that these are the first plasmids captured in estuarine environments may explain this divergent evolution. Anyway, it can be concluded that the *Ria de Aveiro* lagoon might provide such interesting niches to study the IncP-1 plasmid evolution along an estuary displaying different selective forces.

Another point that deserves to be emphasized is related to the high probabilities of the plasmid 8 and 9 to be the same, since they were captured from the same sampling site but different aquatic environment (SML and SSW, respectively), while plasmids coming from exactly the same environment, like 6 and 7 or 9 and 10, are clearly different. That may suggest that the selective pressures are much more important in the evolution than the aquatic environment (SML or SSW). Nonetheless, the low number of captured plasmids strongly disables the establishment of any pattern of plasmid characterization and distribution according to the ecological niches and the particular conditions occurring in each environment.

Finally, the most significant results obtained in this research are the discovery of at least six novel IncP-1 plasmids. As mobile genetic elements with an enormous range of bacterial host, the IncP-1 plasmids represent a huge bacterial diversity source and a highly useful gene spring, since they carry nucleotide sequences that encode traits that allow microbial adaptation to special niches, even more varied due to the extremely different bacterial hosts in which they can be maintained. Therefore, the implication of the discovery of unknown promiscuous plasmids is due to the really important role they play in the HGT (and therefore, in microbial evolution).

The whole description of a novel plasmid may lead to the discovery of unaware genes encoding potentially useful traits (since MGE allows bacterial adaptation, they must carry genes encoding for phenotypic traits that are beneficial for bacteria, and they might be useful for humans too). In addition, the plasmids may be useful as cloning vectors or protein expression vehicles in genetic engineering, which only can become possible from a complete and exhaustive understanding and familiarity with these entities.

Table XIV: Comparison between the seven plasmids obtained from the Ria de Aveiro *lagoon and the previously described model plasmids, pKJK5 and pB10*

	PLASMID NAME	***pKJK5 (IncP-1 ε archetype)***	***1***	***6***	***7***	***pB10 (IncP-1 β archetype)***	***2***	***8***	***9***	***10***
	origin	Soil/manure, Taastrup, Denmark	SML Cais do Sporting (port activities) [selective marker: Tet]	SML Casi do Chegado (industry) [selective marker: Tet]	SML Casi do Chegado (industry) [selective marker: Tet]	Activated sludge from municipal wastewater treatment plant, Braunschweig, Germany	SML Cais do Sporting (port activities) [selective marker: Tet]	SML Casi do Chegado (industry) [selective marker: Hg]	SSW Casi do Chegado (industry) [selective marker: Hg]	SSW Casi do Chegado (industry) [selective marker: Hg]
phenotype traits encoded	**resistance to mercury ions**	no	no	no	no	YES	no	YES	YES	YES
	resistance to tetracycline	YES	YES	YES	YES	YES	YES	no	no	no
	resistance to trimethoprim-sulfamethoxazole	no	no	no	no	no	YES	-	-	-
	resistance to chloramphenicol	no	no	no	no	no	no	-	-	-
	resistance to streptomycin	no	-	-	-	YES	-	-	-	-
	resistance to amoxycilin	no	no	no	no	YES	no	-	-	-
	resistance to amoxicillin and clavulanic acid	no	no	no	no	no	no	-	-	-
	resistance to erythromycin	no	-	-	-	no	-	-	-	-
	resistance to cephalothin	no	no	no	no	no	no	-	-	-
	resistance to aztreonam	no	no	no	no	no	no	-	-	-

* The squares filled with a - indicate not tested traits

6. References

Adamczyk, M.; Jagura-Burdzy, G. 2003. Spread and survival of promiscuous IncP-1 plasmids. Acta Biochimica Polonica 50: 425–453.

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 Research 25: 3389–3402.

Arber, W., 2000. Genetic variation: molecular mechanisms and impact on microbial evolution. FEMS Microbiology Reviews 24: 1–7.

Bahl, M.I.; Burmølle, M.; Meisner, A.; Hansen, L.H.; Sørensen, S.J. 2009. All IncP-1 plasmid subgroups, including the novel ε subgroup, are prevalent in the influent of a Danish wastewater treatment plant. Plasmid 62: 134–139.

Bahl, M.I.; Hansen, L.H.; Goesmann, A.; Sørensen, 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 α, β and δ sub-groups. Plasmid 58: 31–43.

Bale, M.J.; Fry, J.C.; Day, M.J. 1987. Plasmid Transfer between Strains of *Pseudumonas aeruginusa* on Membrane Filters Attached to River Stones. Journal of General Microbiology 133: 3099-3107.

Bergströma, M.; Hermanssona, M.; Dahlberg, C. 2004. Isolation and sequencing of the replication region of plasmid pBFp1 isolated from a marine biofilm. Plasmid 51: 179-184.

Bernardini, M.L.; Mounier, J.; D'hauteville, H,; Coquis-rondont, M.; Sansonetti, P.J. 1989. Identification of *jcsA*, a plasmid locus of *Shigella flexneri* that governs bacterial intra- and intercellular spread through interaction with F-actin. Proceedings of the National Academy of Sciences 86: 3867-3871.

Binh, C.T.; Heuer, H.; Kaupenjohann, M.; Smalla, K. 2008. Piggery manure used for soil fertilization is a reservoir for transferable antibiotic resistance plasmids. FEMS Microbiology Ecology 66: 25–37.

Broughtona, W.J.; Samreya, U.; Stanley, J. 1987. Ecological genetics of Rhizobium meliloti: symbiotic plasmid transfer in the Medicago sativa rhizosphere. FEMS Microbiology Letters 40: 251-255.

Clinical and laboratory standards institute. 2005. Perforamance standards for antimicrobial susceptibility testing, 15th informational supplement. CLSI/NCCLS document M100-S15. Wayne, PA: National Committee for Clinical Laboratory Standards.

Couturier, M.; Bex, F.; Bergquist, P.L.; Maas, W.K. 1988. Identification and Classification of Bacterial Plasmids. Microbiological reviews 52: 375-395.

Cunliffe, M.; Colin, M.J. 2009a. The sea-surface microlayer is a gelatinous biofilm. The ISME Journal 3: 1001–1003.

Cunliffe, M.; Harrison, E.; Salter, M.; Schäfer, H.; Upstill-Goddard, R.C.; Murrell, J.C. 2009b. Comparison and validation of sampling strategies for the molecular microbial analysis of surface microlayers. Aquatic microbial ecology 57: 69–77.

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. Applied and environmental microbiology 63: 4692–4697.

Datta, N.; Hedges, R.W.: Shaw, E.J.; Sykes, R.B.; Richmond, M.H. 1971. Properties of an R factor from *Pseudomonas aeruginosa*. Journal of Bacteriology 108: 1244–1249.

Dominy, C.M.; Deane, S.M.; Rawlings, D.E. 1997. A geographically widespread plasmid from *Thiobacillus ferrooxidans* has genes for ferredoxin-, FNR-, prismane- and NADH-oxidoreductase-like proteins which are also located on the chromosome. Microbiology 143: 3123-3136.

Don, R.H.; Pemberton, J.M. 1981. Properties of six pesticide degradation plasmids isolated from *Alcaligenes paradoxus* and *Alcaligenes eutrophus*. Journal of Bacteriology 145: 681–686.

Dröge, M.; Pühler, A.; Selbitschka, W. 2000. Phenotypic and molecular characterization of conjugative antibiotic resistance plasmids isolated from bacterial communities of activated sludge. Molecular and General Genetics 263: 471-482.

Drönen, A.K.; Torsvik , V.; Goksöyr , J.; Top, E.M. 1998. Effect of mercury addition on plasmid incidence and gene mobilizing capacity in bulk soil. FEMS Microbiology Ecology 27: 381-394.

Faust, R.M.; Abe, K.; Held, G.A.; Iizuka, T.; Bullab, L.E.; Meyers, C.L. 1983. Evidence for plasmid-associated crystal toxin production in *Bacillus thuringiensis* subsp. *israelensis*. Plasmid 9: 98-103.

Forde, A.; Daly, C.; Fitzgerald, G.F. 1999. Identification of Four Phage Resistance Plasmids from *Lactococcus lactis* subsp. *cremoris* HO2. Applied Environmental Microbiology 65: 1540–1547.

Franklin, M.P.; McDonald, I.R.; Bourne, D.G.; Owens, N.J.P.; Upstill-Goddard, R.C.; Murrell, J.C. 2005. Bacterial diversity in the bacterioneuston (sea surface microlayer): the bacterioneuston through the looking glass. Environmental Microbiology 7: 723–736.

Götz, A.; Pukall, R.; Smit, E.; Tietze, E.; Prager, R.; Tschäpe, H.; Van Elsas J.D.; Smalla, K. 1996. Detection and characterization of broad-host-range plasmids in environmental bacteria by PCR. Applied and environmental microbiology 62: 2621–2628.

Gstalder, M.E.; Faelen, M.; Mine, N.; Top, E.M.; Mergeay, M.; Couturier, M. 2003. Replication functions of new broad host range plasmids isolated from polluted soils. Research in Microbiology 154: 499–509.

Haines, A.S.; Jones,K.; Batt, S.M.; Kosheleva , I.A.; Thomas, C.M. 2007. Sequence of plasmid pBS228 and reconstruction of the IncP-1a phylogeny. Plasmid 58: 76–83.

Harada, K.M.; Aso, Y.; Hashimoto, W.; Mikami, B.; Murata, K. 2006. Sequence and analysis of the 46.6-kb plasmid pA1 from *Sphingomonas* sp. A1 that corresponds to the typical IncP-1β plasmid backbone without any accessory gene. Plasmid 56: 11–23.

Heuer, H.; Szczepanowski, R.; Schneiker, S.; Pühler, A.; Top, E.M.; Schlüter, A. 2004. The complete sequences of plasmids pB2 and pB3 provide evidence for a recent ancestor of the IncP-1{beta} group without any accessory genes. Microbiology 150: 3591-3599.

Hill, K.E.; Weightman, A.J.; Fry, J.C.; 1992. Isolation and screening of plasmids from the epilithon which mobilize recombinant plasmid pD10. Applied and Environmental Microbiology 58: 1292–1300.

Hundle, B.; Alberti, M.; Nievelstein, V.; Beyer, P.; Kleinig, H.; Armstrong, G.A.; Burke, D.H.; Hearst, J.E. 1994. Functional assignment of *Erwinia herbicola* Eho10 carotenoid genes expressed in *Escherichia coli*. Molecular Genetics and Genomics 245: 406-416.

Hurst, M.R.; Glare, T.R.; Jackson, T.A.; Ronson, C.W. 2000. Plasmid-located pathogenicity determinants of *Serratia entomophila*, the causal agent of amber disease of grass grub, show similarity to the insecticidal toxins of *Photorhabdus luminescens*. Journal of Bacteriology 182: 5127-5138.

Jencova, V.; Strnad, H.; Chodora, Z.; Ulbrich, P.; Hickey, W.J.; Paces, V. 2004. Chlorocatechol catabolic enzymes from *Achromobacter xylosoxidans* A8. International Biodeterioration and Biodegradation 54: 175–181.

Jobanputra, R.S.; Datta, N. 1974. Trimethoprim R factors in enterobacteria from clinical specimens. Journal of Medical Microbiology 7: 169–177.

Kamachi, K.; Sota, M.; Tamai, Y.; Nagata, N.; Konda, T.; Inoue, T.; Top, E.M.; Arakawa, Y. 2006. Plasmid pBP136 from *Bordetella pertussis* represents an ancestral form of IncP-1beta plasmids without accessory mobile elements. Microbiology 152: 3477-3484.

Kokjohn, T.A.; Miller, R.V. 1994. IncN plasmids mediate UV resistance and error-prone repair in *Pseudornonas aeruginosa* PA0. Microbiology 140: 43-48.

Larkin, M.A.; Blackshields, G.; Brown, N.P.; Chenna, R.; McGettigan, P.A.; McWilliam, H.; Valentin, F.; Wallace, I.M.; Wilm, A.; Lopez, R.; Thompson, J.D.; Gibson, T.J.; Higgins, D.G. 2007. ClustalW and ClustalX version 2. Bioinformatics 23: 2947-2948. Available from:

http://www.ebi.ac.uk/Tools/clustalw2/index.html

Leplae, R.; Hebrant, A.; Wodak, S.J.; Toussaint, A. 2004 ACLAME: A CLAssification of Mobile genetic Elements. Nucleic Acids Research 32: D45-D49. Available from: http://aclame.ulb.ac.be/

Mandelbaum, R.T.; Allan, D.L.; Wackett, L.P. 1995. Isolation and characterization of a *Pseudomonas* sp. that mineralizes the s-triazine herbicide atrazine. Applied Environmental Microbiology 61: 1451–1457.

Mandelbaum, R.T.; Wackett, L.P.; Allan, D.L. 1993. Mineralization of the s-triazine ring of atrazine by stable bacterial mixed cultures. Applied Environmental Microbiology 59: 1695–1701.

Neve, H.; Geis, A.; Teuber, M. 1984. Conjugal Transfer and Characterization of Bacteriocin Plasmids in Group N (Lactic Acid) *Streptococci.* Journal of Bacteriology 157: 833-838.

Ochman, H.; Lawrence, J.G.; Groisman, E.A. 2000. Lateral gene transfer and the nature of bacterial innovation. Nature 405: 299-304.

Oliveira, C.S.; Bresciani, V.; Almeida, A.; Correia, A. 2009. Conjugative gene transfer and BHR plasmids from the SML and subsurface waters: an estuary from Portugal as a case study. 10th International Symposium on Bacterial Genetics and Ecology (BAGECO10 2009), Uppsala, Sweden.

Oliveira, C.S. ; Azevedo, J.; Moura, A. ; Henriques, I.; Correia, A. 2008. Bacterioneuston versus bacterioplankton: culture independent approach to screen for broad host range plasmids, antibiotic resistance and integron gene sequences. International Plasmid Biology Conference (IPBC 2008), Gdansk, Poland.

Prescott, L.M.; Harley, J.P.; Klein, D.A. 2002a. Microbial recombination and plasmids. In: Microbiology. The McGraw-Hill Companies. 5th edition.

Prescott, L.M.; Harley, J.P.; Klein, D.A. 2002b. Microorganism Interactions and Microbial Ecology. In: Microbiology. The McGraw-Hill Companies. 5th edition.

Ramos, I .2009. Comunidade bacteriana cultivável da microcamada superficial estuarina. Mestrado em Microbiologia, Departamento de Biologia, Universidade de Aveiro. Rasmussen, L.D.; Sorensen, S.J. 1998. The effect of longterm exposure to mercury on the bacterial community in marine sediment. Current Microbiology 36: 291–297.

Sanger, F.; Nicklen, S.; Coulson, A.R. 1977. DNA sequencing with chain-terminating inhibitors. Procedings of the National Academy of Science of USA 74: 5463-5467.

Saunders, J.R.; Saunders, V.A.; Pickup, R.W. 1996. The ecology and evolution of bacterial plasmids. In: Pickup, R.W.; Saunders, J.R. Molecular approaches to environmental microbiology. Cambridge (UK): Ellis Horwood. 18-38.

Sayler, G.S.; Hooper, S.W.; Layton, A.C.; King H.J.M. 1990. Catabolic plasmids of environmental and ecological significance. Microbial Ecology 19: 1-20.

Schell, J,; Van Montagu, M. 1977. The Ti-plasmid of *Agrobacterium tumefaciens*, a natural vector for the introduction of nif genes in plants? Basic Life Sciences 9: 159-179.

Schlüter, A.; Szczepanowski, R.; Pühler, A.; Top, E.M. 2007. Genomics of IncP-1antibiotic resistance plasmids isolated from wastewater treatment plants provides evidence forawidely accessible drug resistance gene pool. FEMS Microbiology Reviews 31:449-477.

Schlüter, A.; Heuera, H.; Szczepanowski, R.; Poler, S.M.; Schneiker, S.; Pühler, A.; Top, E.M. 2005. Plasmid pB8 is closely related to the prototype IncP-1β plasmid R751 but transfers poorly to *Escherichia coli* and carries a new transposon encoding a small multidrug resistance efflux protein. Plasmid 54: 135-148.

Schlüter, A.; Heuer, H.; Szczepanowski, R.; Forney, L.J.; Thomas, C.M.; Puhler, A.; Top, E.M. 2003. The 64,508 bp IncP-1beta antibiotic multiresistance plasmid pB10 isolated from a waste-water treatment plant provides evidence for recombination between members of different branches of the IncP-1beta group. Microbiology 149: 3139–3153.

Sen, D.; Yano, H.; Suzuki, H.; Król, J.E.; Rogers, L., Brown, C.J. ; Top, E.M. 2010. Comparative genomics of pAKD4, the prototype IncP-1delta plasmid with a complete backbone. Limnology and Oceanography: Methods 2: 213–225.

Sieburth, J.M. 1983. Microbiological and organic-chemical processes in the surface and mixed layers. In: Liss PS, Slinn WGN (eds). Air-Sea Exchange of Gases and Particles. Reidel Publishers Co: Hingham, MA. 121–172.

Smalla, K.; Haines, A.S.; Jones, K.; Krögerrecklenfort, 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-1 β plasmids with a complex *mer* transposon as the sole accessory element. Applied and environmental microbiology 72: 7253–7259.

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 Microbiology Ecology 42: 165-175.

Sobecky, P.A. 2002. Approaches to investigating the ecology of plasmids in marine bacterial communities. Plasmid 48: 213–221.

Sobecky, P.A. 1999. Plasmid ecology of marine sediment microbial communities. Hydrobiology 401: 9–18.

Sota, M.; Top, E.M. 2008. Horizontal gene transfer mediated by plasmids. In: Lipps, G. (Ed.), Plasmids: Current Research and Future Trends. Caister Academic Press, Norfolk, U.K. 111–181.

Sota, M.; Endo, M.; Nitta, K.; Kawasaki, H.; Tsuda, M. 2002. Characterization of a class II defective transposon carrying two haloacetate dehalogenase genes from *Delftia acidovorans* plasmid pUO1. Applied Environmental Microbiology 68: 2307–2315.

Swofford, D. L. 2003. PAUP: phylogenetic analysis using parsimony, version 4.0b10

Szpirer, C.; Top, E.; Couturier, M.; Mergeay, M. 1999. Retrotransfer or gene capture: a feature of conjugative plasmids, with ecological and evolutionary significance. Microbiology 145: 3321–3329.

Tauch, A.; Schlüter A.; Bischoff, N.; Goesmann, A.; Meyer, F.; Pühler, A. 2003. The 79,370-bp conjugative plasmid pB4 consists of an IncP-1beta backbone loaded with a chromate resistance transposon, the *strA-strB* streptomycin resistance gene pair, the oxacillinase gene *bla NPS-1*, and a tripartite antibiotic efflux system of the resistance-nodulation-division family. Mol Gen Genomics 268: 570–584.

Tennstedt, T.; Szczepanowski, R.; Krahn, I.; Pühler, A.; Schlüter, A. 2005. Sequence of the 68,869 bp IncP-1a plasmid pTB11 from a waste-water treatment plant reveals a highlyconserved backbone, a Tn402-like integron and other transposable elements. Plasmid 53: 218–238.

Thomas, C.M. 2000a. Paradigms of plasmid organization. Molecular Microbiology 37: 485-491.

Thomas, C.M., 2000b. The Horizontal Gene Pool: Bacterial Plasmids and Gene Spread. Harwood Academic Publishers, Amsterdam.

Top, E.M.; Springael, D.; Boon, N. 2002. Catabolic mobile genetic elements and their potential use in bioaugmentation of polluted soils and waters. FEMS Microbiology Ecology 42: 199–208.

Top,E.M.; Holben, W.E.; Forney, L.J. 1995. Characterization of diverse 2,4-dichlorophenoxyacetic acid- degradative plasmids isolated from soil by complementation. Applied and environmental microbiology 61: 1691–1698.

Vedler, E.; Vahter, M.; Heinaru, A.; 2004. The completely sequenced plasmid pEST4011 contains a novel IncP1 backbone and a catabolic transposon harboring *tfd* genes for 2,4-dichlorophenoxyacetic acid degradation. Journal of Bacteriology 186: 7161–7174.

Verdugo P., Alldredge A.L., Azam F., Kirchman D.L., Passow U., Santschi P.H. 2004. The oceanic gel phase: a bridge in the DOM-POM continuum. Marine Chemistry 92: 67–85.

Vincze, T.; Posfai, J.; Roberts, R.J. 2003. NEBcutter: a program to cleave DNA with restriction enzymes. Nucleic Acids Res. 31: 3688-3691. Available from: http://tools.neb.com/NEBcutter2/index.php

Wurl, O.; Holmes, M. 2008. The gelatinous nature of the sea-surface microlayer. Mar ine Chemistry 110: 89–97.

Wurla, O.; Millera, L.; Röttgersb, R.; Vaglea, S. 2009. The distribution and fate of surface-active substances in the sea-surface microlayer and water column. Marine Chemistry 115: 1-9.

Xia, X.S.; Aathithan, S.; Oswiecimska, K.; Smith, A.R.; Bruce, I.J. 1998. A novel plasmid pIJB1 possessing a putative 2,4- dichlorophenoxyacetate degradative transposon Tn5530 in *Burkholderia cepacia* strain 2a. Plasmid 39: 154–159.