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Co-selection of antibiotic and metal(loid) resistance in gram-negative epiphytic bacteria from contaminated salt marshes

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ABSTRACT

The goal of this study was to investigate co-selection of antibiotic resistance in gram-negative epiphytic bacteria. *Halimione portulacoides* samples were collected from metal(loid)-contaminated and non-contaminated salt marshes. Bacterial isolates (n = 137) affiliated with *Vibrio*, *Pseudomonas*, *Shewanella*, *Comamonas*, *Aeromonas* and with Enterobacteriaceae. *Vibrio* isolates were more frequent in control site while *Pseudomonas* was common in contaminated sites. Metal(loid) and antibiotic resistance phenotypes varied significantly according to site contamination, and multiresistance was more frequent in contaminated sites. However, differences among sites were not observed in terms of prevalence or diversity of acquired antibiotic resistance genes, integrons and plasmids. Gene *merA*, encoding mercury resistance, was only detected in isolates from contaminated sites, most of which were multiresistant to antibiotics. Results indicate that metal(loid) contamination selects for antibiotic resistance in plant surfaces. In salt marshes, antibiotic resistance may be subsequently transferred to other environmental compartments, such as estuarine water or animals, with potential human health risks.

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1. Introduction

The increasing incidence of antibiotic resistance among bacteria has reduced the efficacy of these drugs, constituting a significant threat to public health. Following the rapid dissemination registered in hospital settings, the environment is progressively recognized as an important reactor for the evolution and spread of resistance (Baquero et al., 2008; Finley et al., 2013). Contaminants, other than antibiotics, may favor antibiotic-resistant bacteria proliferation and promote horizontal transfer of resistance genes (Gaze et al., 2005; Tacão et al., 2012). Metal contamination is allegedly a main selector of antibiotic resistance (Bednorz et al., 2013; Stepanauskas et al., 2006). Metals concentration in the environment was found to positively correlate with antibiotic resistance genes abundance (Ji et al., 2012; Knapp et al., 2011). This is of great concern since metals can be found in elevated concentrations in many environmental compartments (Baborowski et al., 2012; Nicholson et al., 2003) and are not subjected to degradation, thus providing a persistent selective pressure.

The co-selection effect (reviewed by Baker-Austin et al., 2006 and Seiler and Berendonk, 2012) relates to: (i) co-resistance, when resistance mechanisms to metals and antibiotics are encoded in the same mobile genetic element; or (ii) cross-resistance, *e.g.*, the same mechanism confers resistance simultaneously to metals and antibiotics. For instance, genes conferring resistance to mercury and antibiotic resistance genes have been detected in the same plasmids (McIntosh et al., 2008; Parkhill et al., 2001) and transposons (Parkhill et al., 2001; Wireman et al., 1997). On the other hand, efflux mechanisms able to export metals and antibiotics have been reported as examples of cross-resistance (Aendekerk, et al., 2002; Nishino et al., 2007).

Despite its public health relevance, many uncertainties remain on the contribution of metal(loid)s for antibiotic resistance selection. More studies are needed to expose environmental hotspots where co-selection is more prone to occur. The hypothesis that bacterial communities in metal(loid)-contaminated environments enclose a more diverse and mobile antibiotic resistance gene pool needs further confirmation.

Estuarine salt marshes are recipients of contaminants resulting from human and industrial activities (Canário et al., 2007; Oliveira et al., 2014). Salt marsh plants can sequestrate metal(loid)s from sediments, and accumulate both in the root and aerial parts (Anjum et al., 2014; Canário et al., 2007). Plant surfaces offer a protected and nutrient-rich environment for bacteria growth, being inhabited by large population of bacteria in close proximity, possibly forming biofilm-like structures (Lindow and Brandl, 2003). As a consequence, plant surfaces represent potential hotspots for horizontal gene transfer (Aminov, 2011; Bezanson et al., 2008). The formation of biofilms may be facilitated by metal exposure that stimulates extracellular polymeric substances production (Kidambi et al., 1995).

Given the mentioned conditions, plant surfaces in metal(loid) contaminated salt marshes may constitute a niche for antibiotic resistance

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selection driven by metal(loid)s. The aim of this study was to assess co-selection of metal(loid) and antibiotic resistance traits in gram-negative epiphytic bacteria from plants collected in metal(loid)-contaminated and non-contaminated salt marshes. Occurrence and diversity of mobile genetic elements (*i.e.*, plasmids and integrons) and resistance genes was also inspected.

2. Material and methods

2.1. Study sites and sampling

Plant samples were collected from salt marshes located in *Ria de Aveiro*, a multi-estuarine ecosystem in the Northwest coast of Portugal. The plant species, *Halimione portulacoides*, was chosen due to its wide distribution and ecological relevance (Anjum et al., 2014). Also, this plant is known for its capacity to accumulate metals and to perform metal remediation (Anjum et al., 2014; Canário et al., 2007).

Composite samples were collected from leaves, stems, flowers and roots, at low tide, in three sites designated as C (40°38′05″N 8°39′38″W), B (40°43′48″N 8°36′45″W) and E (40°43′17″N 8°38′16″W). Site C was chosen as control site, and sites B and E were chosen as metal(loid) contaminated locations, according to previous studies (Válega et al. 2008). Sites B and E are located in a confined area in *Ria de Aveiro* that was subjected to metal-contaminated industrial discharges for four decades (Pereira et al., 1998).

2.2. Quantification of metal(loid)s

Composite samples of sediment (2 g fresh weight) from each site were used for metal(loid) quantification, in triplicate. Chromium (Cr), nickel (Ni), copper (Cu), zinc (Zn), arsenic (As) and mercury (Hg) were quantified. Samples were subjected to acid digestion with concentrated nitric acid overnight at 115 °C (Figueira and Freitas, 2013), and analyzed by Inductively Coupled Plasma Mass Spectrometry according to International Standard ISO 17294 (ISO, 2005) by a certified laboratory at the University of Aveiro. The trace elements analyzed (As, Cr, Cu, Hg, Ni and Zn) were selected based on reported contamination level in Ria de Aveiro (Anjum et al., 2014; Válega et al., 2008) and/or on identified linkages between resistance to these metal(loid)s and antibiotic resistance (Bednorz et al., 2013).

2.3. Bacterial isolation, typing and identification

Plants were inspected to remove decaying plant material. Roots were vigorously shaken to remove sediment. Plant tissues were washed with phosphate-buffered saline. The resulting solution was then filtered in cellulose ester filters (0.45 μm pore size). Membranes were placed on MacConkey agar plates and incubated at 37 °C for 16 h. Individual colonies were purified and stored in 20% glycerol at −80 °C.

Genomic DNA was isolated as previously described (Henriques et al., 2006) and BOX-PCR was used to type all isolates (Alves et al., 2004). Isolates with different band patterns were identified by 16S rRNA gene sequencing analysis with primers and PCR conditions previously described (Henriques et al., 2006). PCR products were purified using the DNA Clean & Concentrator Kit (Zymo Research, USA) and used as template in the sequencing reactions. Similarity searches were performed with the BLAST software (Altschul et al., 1990) at the National Center of Biotechnology Information website.

2.4. Antibiotic susceptibility testing

Antimicrobial resistance patterns were determined by the agar disc diffusion method on Mueller–Hinton agar, against 16 antibiotics. Discs (Oxoid, United Kingdom) contained the following agents: amoxicillin (10 μg), amoxicillin/clavulanic acid (20 μg/10 μg), ampicillin (10 μg), aztreonam (30 μg), cefepime (30 μg), cefotaxime (30 μg), ceftazidime (30 μg), cephalothin (30 μg), ciprofloxacin (5 μg), chloramphenicol (30 μg), gentamicin (10 μg), imipenem (10 μg), kanamycin (30 μg), nalidixic acid (30 μg), sulfamethoxazole/trimethoprim (25 μg) and tetracycline (30 μg). After 24 h of incubation at 37 °C, organisms were classified as sensitive, intermediate, or resistant according to the Clinical Laboratory Standards Institute guidelines (CLSI, 2012). The reference strain *Escherichia coli* ATCC 25922 was included as quality control.

2.5. Metal(loid) susceptibility testing

Minimal inhibitory concentrations (MICs) were determined in Luria-Broth (LB) medium supplemented with As (5, 10 and 20 mM As as Na_2AsO_4), Cr (1 and 5 mM Cr as $CrCl_3 \cdot 6H_2O$), Cu (1 and 5 mM Cu as $CuSO_4 \cdot 5H_2O$), Hg (0.1, 0.5 and 5 mM Hg as $HgCl_2$), Ni (1, 2 and 5 mM Ni as $NiSO_4 \cdot 6H_2O$) and Zn (0.5, 1, 2, 5 mM Zn as $ZnCl_2$). Metal(loid) stock solutions (125 mM) were prepared in distilled water and sterilized by filtration. Experiments were prepared in triplicate in 96-well microtiter plates. Each well contained 100 μL of LB supplemented with metal(loid)s and was inoculated with a 10 μL aliquot of cells from a culture at an OD595 of 0.1. Growth was measured by optical density at 595 nm with a microplate reader (BioRad, USA), after 5 days incubation at 37 °C and 110 rpm. The reference strain *E. coli* ATCC 25922 was included for quality control. The MIC of each metal(loid) was recorded as the lowest concentration at which the isolate did not grow. Since there are no standard interpretative criteria for the classification of bacterial isolates as susceptible or tolerant to metal(loid)s, thresholds were defined based on previous studies (Malik and Aleem, 2011; Nies, 1999).

2.6. PCR amplification of resistance genes and integrons

The presence of genes encoding resistance against beta-lactams (bla_{TEM}, bla_{SHV}, bla_{IMP}, bla_{VIM}, bla_{KPC}, bla_{GES}, bla_{VEB}, bla_{PER}, $bla_{CTX\text{-}M}$, $bla_{OXA\text{-}48}$), tetracycline (*tetA*, *tetB*, *tetD*, *tetE*, *tetG*, *tetM*), sulfonamides (*sul1*, *sul2*, *sul3*), quinolones (*qnrA*, *qnrB*, *aacA4-cr*), and aminoglycosides (*aadA1*, *aadA2*, *strA*, *strB*), was detected by PCR using primers and conditions listed in Table S1. The presence of the *mer* operon, conferring tolerance to Hg, was assessed by amplifying a 933 bp fragment of the *merA* gene as described by Deredjian et al. (2011). PCR screening was performed for integrase genes *intI*1 and *intI*2 and variable regions of integrase-positive isolates were further analyzed by PCR (Henriques et al., 2006).

2.7. Plasmid procedures

The presence of plasmid DNA in bacterial isolates was confirmed using several purification methods: alkaline lysis with SDS (Sambrook and Russel, 2001), Qiagen Plasmid Mini-kit (Qiagen GmbH, Germany), EZNA Plasmid DNA mini Kit II (Omega Bio-tek, USA) and NZYMiniprep plasmid DNA (NZYTech, Portugal). Diversity of plasmids was evaluated by plasmid restriction analysis using 5 U of *Pst*I (CTGCA↓G) and 5 U of *Bst*1770I (GTA↓TAC), according to the manufacturer's instructions (Fermentas, Lithuania). Restriction patterns were visualized in 0.8% agarose gels. Electrophoresis was run at 40 V for 3 h and gels were stained using ethidium bromide. Restriction patterns were compared using GelCompar II software (Applied Maths, Sint-Martens-Latem, Belgium).

For replicon typing, detection of FIA, FIB, FIC, HI1, HI2, I1-Ig, L/M, N, P, W, T, A/C, K, B/O, X, Y, FrepB, and FIIA replicons was performed by PCR, using primers and conditions described by Carattoli et al. (2005). Positive results were confirmed by repeating the positive PCR as a simplex PCR and by sequencing.

2.8. Statistical analysis

One-way ANOVA combined with post hoc Tukey's Honest Significant Differences method were used to assess differences in metal(loid)

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