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Novel gene cassettes and integrons in antibiotic-resistant bacteria isolated from urban wastewaters

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Abstract

In this study, the occurrence and diversity of integrons were evaluated in 697 isolates belonging to *Enterobacteriaceae* and *Aeromonas* spp. isolated from urban wastewaters. Screening of integrons was performed by dot blot hybridization and *intI*-positive strains were further characterized. The global prevalence of integrons was 3.73%. Three new gene cassettes were identified: a novel *aadA* variant (*aadA17*), a gene putatively involved in cell signaling (*dcyA*) and an open reading frame of unknown function interrupted by a novel insertion sequence (*orfER.17*::IS*As12*). In total, thirteen different gene cassette arrays were detected, 4 representing novel integrons: *intI1-dcyA-tniC*, *intI1-orfER.1.7*::IS*As12-aadA13-qacE*Δ*1-sul1*, *intI1-aacA4-catB3-bla*$_{OxA-10}$*-aadA1-qacE*Δ*1-sul1* and *intI1-catB8-aadA17-qacE*Δ*1-sul1*. Approximately 80% of strains were resistant to at least 3 antibiotics of different classes. The presence of novel integron structures in treated effluents suggests that domestic wastewaters may favor the formation of novel combinations of gene cassettes. Moreover, the high prevalence of multiresistant strains highlights the urgent need to employ effective means of effluent disinfection to avoid dissemination of antibiotic-resistant bacteria.

Keywords: Wastewaters; Integrons; Multiresistance; Mobile genetic elements; *Aeromonas*; *Enterobacteriaceae*

1. Introduction

Urban wastewater treatment usually consists of a combination of physical, chemical and biological procedures to eliminate or reduce suspended solids and organic matter loads from effluents, without damage to natural environments (Pescod, 1992).

Recycling of treated wastewater and sludge in agriculture is encouraged by European Commission directives (86/278/EEC), owing to the presence of high concentrations of nutrients that reduces the requirements for inorganic fertilizers and consequently crop production costs. However, high nutrient loads and intensive selective pressures imposed by detergents and pharmaceuticals make wastewater treatment plants (WWTP) favorable sites for horizontal gene transfer events (Moura et al., 2007, 2010; Schluter et al., 2007b; Xu et al., 2007). It has been shown that antibiotics (Xu et al., 2007) and antibiotic-resistant bacteria (Moura et al., 2007) continue to persist in disturbing amounts in treated effluents due to incomplete metabolism of excreted antibiotics. Consequently, growing concern has arisen regarding the occurrence and diversity of mobile genetic elements in wastewaters and the possible role of these environments in the dissemination of resistance and/or virulence genetic traits which may reach natural waters, soils and eventually the food chain (Martinez, 2009; Moura et al., 2010).

Integrons are genetic systems that allow bacteria to capture and express gene cassettes. They typically consist of an *intI* gene encoding for an integrase that catalyzes the incorporation or excision of gene cassettes by site-specific recombination, a recombination site *attI* and one or two promoters responsible for the expression of inserted gene cassettes (Cambray et al., 2010). Integrons were first reported in clinical isolates in the

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1980s and continue to be extensively studied in clinical environments, due to their association with other mobile genetic elements and multiresistance phenotypes (Hall et al., 1999; Leverstein-van Hall et al., 2002). However, in the last decade special attention has been given to integrons from natural environments in order to gather information on their ecology and diversity, and to understand their role in bacterial adaptation. Evidence that stress response may trigger the expression of *intI* (Guerin et al., 2009) and gene cassettes (Michael and Labbate, 2010) has been recently reported, suggesting that integrons may constitute important adaptive systems in bacterial evolution.

Integron-related sequences have been found in a wide diversity of bacterial hosts, most of them (~30%) belonging to γ-Proteobacteria (Moura et al., 2009). The wide dispersion of integrons is probably a consequence of their association with other mobile genetic elements, which not only increases the genetic traits harbored but also the mobilizing events, contributing to the spread of such elements in the environment. Their diversity and abundance may also be related to the kind of selective pressures they encompass (Wright et al., 2008).

In a previous study, we investigated the prevalence of integrons among enterobacteria and aeromonads isolated from a WWTP receiving waters from a slaughterhouse (Moura et al., 2007). The presence of integrase-carrying bacteria in the slaughterhouse's wastewaters was determined to be 35%, a considerably high value compared to other aquatic environments (Rosser and Young, 1999; Henriques et al., 2006b). Moreover, nearly 50% of strains were resistant to 5 or more antibiotics, once again drawing attention to the misuse of antibiotics in veterinary therapeutics and for the role of WWTP as hotspots for horizontal gene transfer.

The aim of the present study was to evaluate the occurrence and diversity of integrons in water samples from an urban WWTP in order to assess the influence of the type of effluent on the prevalence of integrons and on the diversity of their gene cassettes among *Enterobacteriaceae* and *Aeromonas* spp.

2. Materials and methods

2.1. Sampling and bacterial isolation

Sampling was performed in November 2007 in an activated sludge plant receiving domestic effluents, located in Ermesinde in the north of Portugal. Water samples were collected in 0.5 l autoclaved bottles at 5 points throughout the WWTP: raw water (RW), a primary decantation tank (PD), an aeration tank (AT), sludge recirculation (SR) and final effluent (FE). Biochemical oxygen demand (BOD_5), chemical oxygen demand (COD), total suspended solids (TSS) and pH were determined by standard methods (Clescerl et al., 1998).

Serial decimal dilutions of water samples were prepared in 0.9% NaCl and filtered through 0.45 μm-pore size cellulose sterile filters (Pall Life Sciences, MI, USA). Filters were placed onto glutamate starch phenol red (GSP) agar plates (Merck, Darmstadt, Germany) selective for *Aeromonas* and MacConkey agar for *Enterobacteriaceae* (Merck, Darmstadt, Germany). Duplicate sets of plates were incubated for 24 h at 30 °C and 37 °C. All individual colonies were picked from the following dilutions: 10^{-4} and 10^{-5} in raw waters, 10^{-3} and 10^{-7} in a primary decantation tank, 10^{-4} and 10^{-5} in an aeration tank, 10^{-6} and 10^{-8} in sludge recirculation and 10^{-2} and 10^{-2} in final effluent, for MacConkey and GSP agar, respectively.

2.2. Integron screening and identification of integrase-positive isolates

Oligonucleotides used during this study are listed in Table 1. Bacterial colonies were screened for the presence of *intI1*, *intI2* and *intI3* genes by dot blot hybridization, as described previously (Moura et al., 2007). Colonies giving positive hybridization signals were further purified by continuing isolation in MacConkey and GSP agar. Molecular typing of integrase-positive isolates was carried out by REP-PCR (Versalovic et al., 1991). Identification of isolates representing different REP profiles was achieved by amplification and sequencing of the 16S rRNA gene (Lane, 1991). Identification of *Aeromonas* isolates to the species level was achieved by partial amplification (~950 bp) and sequencing of the *gyrB* gene (Yanez et al., 2003). Sequence similarity searches were conducted using BLAST software (Altschul et al., 1997) and nucleotide sequences were aligned by CLUSTAL X (Thompson et al., 1997). Phylogenetic trees were constructed by the neighbor-joining method (Saitou and Nei, 1987) using MEGA version 4.0 (Tamura et al., 2007).

2.3. Integron characterization and genetic location

Integron characterization was carried out through PCR amplification with primers targeting class 1 and class 2 integron variable regions (Levesque et al., 1995; White et al., 2001), as previously described (Moura et al., 2007). To determine the structure of the 3′-segment of class 1 integrons, the presence of *qacE*, *sul1*, *sul3*, IS*CR1* (orf513) and *tniC* (*tniR*) genes was also tested (Table 1).

Strains that failed to amplify integron variable regions with primers 5′CS/3′CS (class 1 integrons) and hep74/hep51 (class 2 integrons) were further subjected to amplification using different combinations of primers (Table 1) and the Extensor Long PCR Master Mix (ABgene, UK) according to manufacturer's instructions.

To determine integron location, total genomic DNA and plasmid DNA were purified, separated by electrophoresis and transferred onto positive-charged membranes, as described previously (Moura et al., 2007). Southern blots were probed using *intI*-labeled fragments, as described previously (Moura et al., 2007).

2.4. Conjugative gene transfer

Strains containing plasmid-borne integrons were used as donors in mating assays using rifampicin- and kanamycin-resistant *Escherichia coli* CV601-GFP as recipient strain