



Abundance of class 1–3 integrons in South Carolina estuarine ecosystems under high and low levels of anthropogenic influence



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ABSTRACT

The impact of human activity on the spread of antibiotic resistant bacteria throughout coastal estuarine ecosystems is not well characterized. It has been suggested that laterally transferred genetic agents, such as integrons, play a role in the spread of resistant bacteria throughout ecosystems. This study compares the distribution of three integron classes throughout a coastal estuarine ecosystem. To determine integron distribution patterns, DNA was extracted from sediment and water collected at seven sites throughout two estuaries with different levels of anthropogenic input and integrons analyzed using quantitative PCR. The data show that while all three integron classes are present, the relative abundance is different, with class 2 integrons significantly elevated in areas of high anthropogenic input and class 1 integrons elevated in areas of low input. Our results provide a foundation for using integron gene distribution as a biomarker of urban impact on antibiotic resistance gene flow and ecosystem health.

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1. Introduction

It has been estimated that 40% of the global population lives within 100 km of coastlines (UNEP, 2006), with 33 of the 50 world's largest cities located in coastal zones (Dean, 1997). A similar pattern has been observed in the United States where half of the population resides within 80 km of the coastline (Crossett et al., 2004), with this proportion projected to increase to 70% by the year 2025 (Hinrichsen, 1998). Increases in coastal migration and changes in land use patterns have been linked to the degradation of many estuarine ecosystems resulting in reduced resources provided from these areas for sustainability and recreation (Comerio et al., 1996; Vernberg et al., 1996; Bricker et al., 1999; EPA, 2004; Dauer et al., 2000; Paul et al., 2002; Nelson et al., 2005; Van Dolah et al., 2008). In addition to the chemical and biological agents commonly associated with ecosystem degradation, it has been suggested that bacterial antibiotic resistance genes be included as an emerging contaminant of great public health concern that may also serve as bioindicators of ecosystem quality (Pruden et al., 2006). However, before antibiotic resistance genes

can be used as an indicator of coastal estuarine ecosystem health, it is important to examine the anthropogenic impact on microbial resistance gene patterns.

While selective pressure may result in independent evolution of genetic point mutations, giving rise to bacterial antibiotic resistance genes, it is the location of these genes within mobile elements that allows them to be readily and quickly transferred horizontally throughout an ecosystem (Mazel, 2006; Cambray et al., 2010). Integrons are one such microbial mobile element that plays a role in the dissemination of antibiotic resistance genes. Integrons are genetic elements often associated with pathogenic and commensal bacteria that confer the ability to capture and express exogenous and promoterless gene cassettes, which encode a number of adaptive functions including antibiotic resistance (Mazel, 2006; Boucher et al., 2007; Rodriguez-Minguela et al., 2009). Four elements define an integron structure: a tyrosine recombinase or integron integrase (*intI*) which is responsible for driving the gene cassette insertion; a recombination site (*attI*); a promoter (Pc) located upstream of the cassette region and responsible for the expression of gene cassettes; and the actual gene cassettes, many of which confer resistance to a wide range of antibiotics (Jones et al., 2003; Diaz-Mejia et al., 2008).

Among these elements, differences in the *intI* gene sequence are used to differentiate unique integron classes (Sá et al., 2010). While integrons are phylogenetically diverse, three major groups (classes 1–3) are known to be associated with horizontally transferred elements that contribute significantly to the spread of antibiotic resistance (Partridge et al., 2009). Class 1 integrons are typically

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linked to replicative Tn21 transposons and are the most abundant integron class found in clinical isolates (Rosser and Young, 1999; Mazel, 2006; Solberg et al., 2006; Diaz-Mejia et al., 2008; Wright et al., 2008). More than 80 different gene cassettes of class 1 integrons have been described and shown to confer resistance to a wide range of antibiotics such as β -lactams, fluoroquinolones, aminoglycosides, chloramphenicol, trimethoprim, streptothricin, rifampin, erythromycin, fosfomycin, lincomycin, and antiseptics and disinfectants (Rowe-Magnus and Mazel, 2002; Fluit and Schmitz, 2004). Class 2 integrons have been reported most often in isolates within the *Enterobacteriaceae* family and less frequently in other isolates affiliated with the beta, gamma, and epsilon subdivisions of the *Proteobacteria* (Fluit and Schmitz, 2004; Ramirez et al., 2005; Rodriguez-Mingueta et al., 2009). These integrons are associated with nonreplicative Tn7 transposons with up to 12 different gene cassette arrays described as conferring resistance to aminoglycosides, chloramphenicol, trimethoprim, streptothricin (Biskri and Mazel, 2003; Ramirez et al., 2005; Barlow and Gobius, 2006; Mazel, 2006). As compared to class 1 and 2, less is known about class 3 integrons which have been described as rare and found only in a limited number of isolates including *Serratia marcescens*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Pseudomonas putida*, *Alcaligenes xylosoxidans*, and *Delftia* spp. (Collis et al., 2002; Correia et al., 2003; Shibata et al., 2003; Fluit and Schmitz, 2004; Xu et al., 2007). Currently, gene cassettes within class 3 integrons have been shown to confer resistance to ceftazimide, sulbactam, and cefeprozone (Shibata et al., 2003; Fluit and Schmitz, 2004).

These integron classes, commonly containing antibiotic resistance genes, have been documented in a wide variety of environments such as hospitals, soils, sediments, aqua culture facilities, oyster beds, and wastewater treatment plants (Schmidt et al., 2001; White et al., 2001; Roe et al., 2003; Solberg et al., 2006; Rao et al., 2006; Diaz-Mejia et al., 2008; Gillings et al., 2008; Ozgumus et al., 2009; Barkovskii et al., 2010). Most of these studies have been conducted on bacterial isolates using culture-dependent methods or focused on a particular integron class, thus underestimating overall integron abundance and complexity within these ecosystems. However, a recent study surveyed the presence of class 1–3 integrons within oyster beds and found that integron distribution may be correlated with agricultural and municipal run-offs (Barkovskii et al., 2010). This study focused on an area of low human population density and high agricultural use and found a higher incidence of class 3 integrons within oysters as compared to class 1 and 2.

Currently, no quantitative culture-independent studies have simultaneously compared the difference in the distribution of the three major classes of integrons within highly urbanized and pristine coastal estuarine ecosystems. To determine the urban impact on integron distribution, we used quantitative PCR to examine the abundance of class 1–3 integrase genes in sediments and water collected from sites within Charleston Harbor, an area of high anthropogenic input, and a less impacted site listed within NOAA's National Estuarine Research Reserve System. For further comparison, we also examined the distribution of integrons within a wastewater treatment plant (WWTP) that discharges into Charleston Harbor. A better understanding of the anthropogenic impact on integron distribution will provide insight on one possible mechanism for the spread of antibiotic resistant genes throughout an ecosystem and may provide an additional indicator of overall ecosystem health with potential public health consequences.

2. Materials and methods

2.1. Coastal estuarine ecosystem study sites

The urban-impacted coastal estuarine ecosystem examined in this study is located along the southern coast of South Carolina,

USA and has an average yearly salinity of 27 psu and surface water temperature of 22.6 °C (Fig. 1A). The Charleston Harbor estuary covers approximately 3300 km² and is composed of three tributaries: the Cooper, Wando, and Ashley Rivers (Yassuda et al., 2000). As the second largest container port on the East Coast, this ecosystem supports a rapidly growing economy with a concurrent 7% annual urban area expansion rate (Allen and Lu, 2003) consisting of mixed residential, urban, and light industrial use. This area also supports a growing population through its use as a recreational site for swimming, boating, and fishing. Sediment and water sampling sites were located in the lower portion of the Charleston Harbor estuary at 32°46'2.99"N; 79°56'22.92"W (site 2), 32°46'56.56"N; 79°57'36.68"W (site 3), 32°46'0.44"N; 79°54'23.75"W (site 4), 32°47'38.94"N; 79°54'51.37"W (site 5), 32°45'13.71"N; 79°52'18.22"W (site 6), and 32°46'13.72"N, 79°52'31.60"W (site 7).

As a non-urbanized control site, sediment and water samples were also collected near the Oyster Landing monitoring station at North Inlet, Winyah Bay, SC (Fig. 1B, site 8; 33°21'0.36"N; 79°11'24.18"W). North Inlet is located ~96 km northeast of Charleston Harbor and is a bar-built estuary bounded by Debidue Island to the northeast and North Island to the southeast. This site is part of the NOAA's National Estuarine Research Reserve System (NERRS) and while experiencing similar environmental conditions as Charleston Harbor, it is less developed resulting in reduced urban influence.

Furthermore, as a potential point source of contamination in Charleston Harbor, we also examined a wastewater treatment facility located in Charleston, SC (Fig. 1A, site 1; 32°45'36.84"N; 79°56'57.89"W). With an optimal operating capacity of 136 million l day⁻¹ of treated wastewater, the facility uses primary treatment involving physical removal of debris and large particles from the wastewater prior to secondary biological treatment and final chlorination. The outfall (site 2) is located 1219.20 m from the shore in Charleston Harbor.

2.2. Sample collection

Charleston Harbor samples were collected during a flood tide in August 2010. Three one-liter seawater samples were taken at a depth of 2 m using a Niskin bottle and stored in sterile polyethylene bottles for further processing. Three sediment samples (~1 kg) were collected at the same geographical sites using an Ekman dredge and stored in one-liter sterile polyethylene bottles. The Ekman dredge was rinsed with nitric acid, acetone, and de-ionized water between stations. The same protocol was employed to collect seawater and sediment samples in the Oyster landing station in North Inlet. Water quality parameters were measured at each site using a YSI 556 Multiparameter System with probe placed mid-water column.

Wastewater samples were collected immediately prior to Charleston Harbor sampling using sterile polyethylene one-liter bottles ($n = 2$) from 3 different stages of the wastewater treatment process: raw sewage (RS), activated sludge (AS), and principal effluent (PE). Once samples were collected, they were immediately stored on ice and transported to the laboratory for processing and storage (within 3 h).

2.3. DNA extraction

Seawater and sediment samples were extracted using the Ultra-Clean Soil DNA kit (MoBio, Carlsbad, CA) following the manufacturer's instructions, but due to low cell density, 1000 ml of seawater samples were first concentrated by filtration using a 0.22 μ m Sterivex filter unit (Millipore Corporation, Billerica, MA) then washed with phosphate buffer solution to remove the cells from the filter before DNA extraction. DNA extracted from

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