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Multiresistant *Enterobacteriaceae* with class 1 and class 2 integrons in a municipal wastewater treatment plant

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ARTICLE INFO

Article history:
Received 6 November 2011
Received in revised form
8 March 2012
Accepted 20 March 2012
Available online 2 April 2012

Keywords:
Antibiotic resistance
Integrons
Gene cassettes
Wastewater

ABSTRACT

In this study, 1832 strains of the family *Enterobacteriaceae* were isolated from different stages of a municipal wastewater treatment plant, of which 221 (12.1%) were *intI*-positive. Among them 61.5% originated from raw sewage, 12.7% from aeration tank and 25.8% from the final effluent. All of the *intI*-positive strains were multiresistant, i.e. resistant to at least three unrelated antimicrobials. Although there were no significant differences in resistance range, defined as the number of antimicrobial classes to which an isolate was resistant, between strains isolated from different stages of wastewater treatment, for five β-lactams the percentage of resistant isolates was the highest in final effluent, which may reflect a selective pressure the bacteria are exposed to, and the possible route of dissemination of β-lactam resistant strains to the corresponding river. The sizes of the variable part of integrons ranged from 0.18 to 3.0 kbp and contained up to four incorporated gene cassettes. Sequence analysis identified over 30 different gene cassettes, including 24 conferring resistance to antibiotics. The highest number of different gene cassettes was found in bacteria isolated from the final effluent. The gene cassettes were arranged in 26 different resistance cassette arrays; the most often were *dfrA1-aadA1*, *aadA1*, *dfrA17-aadA5* and *dfrA12-orfF-aadA2*. Regarding the diversity of resistance genes and the number of multiresistant bacteria in the final effluent, we concluded that municipal sewage may serve as a reservoir of integron-embedded antibiotic resistance genes.

1. Introduction

The main factor in the spread of drug resistance has been the ability of bacteria to acquire and disseminate exogenous genes *via* mobile elements such as conjugative plasmids and transposons. Bacterial resistance has been primarily analyzed in pathogenic strains within hospital settings (Daikos et al., 2007; Gillings et al., 2008). However, it is known that bacteria participate in common gene pool and transfer of genes among bacteria residing in different habitats has been proved (Dröge et al., 1998; Thomas and Nielsen, 2005; Schlüter et al., 2007). Transmission between humans and the environment has been shown for nosocomial *Enterococcus faecium*, which was passed from patients in hospitals to hospital and then urban sewage, and further *via* treatment plants to surface water and possibly back to humans (Iversen et al., 2004). There has been also an evidence for transfer of resistance to human commensal bacteria and pathogens, and gene transfer in human intestine (Salyers et al., 2004; Pallecchi et al., 2010). The resistance can persist long in bacterial communities even if the selective pressure imposed by antibiotic usage is reduced. The reason for this is that displacement of antibiotic-resistant

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0043-1354/$ – see front matter © 2012 Elsevier Ltd. All rights reserved.
doi:10.1016/j.watres.2012.03.037

population with susceptible one takes long time or does not exist, due to slow intrinsic reversal, compensatory evolution and cost-free resistances (Anderson and Hughes, 2010).

Urban wastewater treatment plants (WWTPs) represent important reservoirs of human and animal microorganisms, in which antibiotic resistant bacteria can persist in the final effluent and enter environment with treated sewage (Reinthaler et al., 2003; Guo et al., 2011; Figueira et al., 2011; West et al., 2011). The features of this environment, like the high number of bacteria and the presence of the trace concentrations of antibiotics, may facilitate the bacterial conjugation (Baquero et al., 2008; Segura et al., 2009; Courvalin, 2008). It is generally believed that WWTPs serve as important reservoirs for resistance genes situated on mobile genetic elements and that municipal WWTPs represent hot-spots for horizontal gene transfer. Hence, with their key position at the interface between human activities and the environment, WWTPs can be used to control antimicrobial resistance (Dröge et al., 2000; Tennstedt et al., 2003; Schlüter et al., 2007; Ghosh et al., 2009; Ramsden et al., 2010).

The resistance genes located on transposons and plasmids can be a part of genetic platforms called integrons (Hall and Collis, 1995). Integrons are natural systems mediating the capture of gene cassettes that mostly code for antibiotic resistance. The integron platform covers DNA fragment that consists of an integrase gene of the tyrosine recombinase family, primary recombination site called the *attI*, and a P_C promoter that directs transcription of the captured genes. Integron-encoded integrase can recombine discrete units of circularized DNA known as gene cassettes. As the integron system has the ability to create novel combinations of resistance genes, it may facilitate the evolution of multidrug resistant bacteria. Five classes of resistance integrons have been defined based on the genetic polymorphism of the integrase genes (Cambray et al., 2010). They are responsible for spreading of multidrug resistance, with class 1 being most ubiquitous among resistant bacteria and considered to play the main role in the emergence and wide dissemination of resistance genes, especially among Gram-negative clinical isolates (Leverstein-van Hall et al., 2003).

The aim of the study was to determine the occurrence and characteristics of integrons in bacteria of the family *Enterobacteriaceae* in the sewage in Central Wastewater Treatment Plant in Koziegłowy near Poznań, Poland, and to evaluate antimicrobial resistance of integron-harbouring isolates at different stages of wastewater treatment.

2. Material and methods

2.1. Sampling and strain isolation

Sewage samples were taken from a municipal wastewater treatment plant four times between December 2008 and February 2010. The samples were taken from (i) raw sewage in primary sedimentation tank, (ii) aeration tank, and (iii) final effluent of treated wastewater. In each sampling event, the samples were taken simultaneously from the three sites. The samples were collected in sterile containers at the depth of 0.3 m and the distance of 1 m from the side of the respective sampling sites. The total number of culturable bacteria and the number of coliforms were determined by plate counts. Serial decimal dilutions of the samples were prepared in 0.9% NaCl, inoculated onto Brilliance™ *E. coli*/Coliform Selective Agar (Oxoid) and incubated overnight at 37 °C. Single colonies were picked from the medium and used in further experiments. Identification of bacteria was done with API 20E kit (bioMérieux), dedicated to identifying *Enterobacteriaceae* and other G(−) rods with the use of biochemical tests.

2.2. Clonal analysis by ERIC PCR

ERIC (enterobacterial repetitive intergenic consensus sequence) PCR fingerprinting was applied to determine clonal relatedness of the isolates. The ERIC PCR was done according to Versalovic et al. (1991) with primers ERIC 1 and ERIC 2. The PCR products were separated in agarose gel, stained with ethidium bromide and digitalized with Bio-Print v. 99 gel documentation system (Vilbert-Lourmat). The electrophoretic patterns were analyzed by using GelCompar II 3.5 software (Applied Maths) with internal and external normalization. Optimization and band position tolerance were set at 1%. Similarity between fingerprints was calculated with the Dice coefficient. Cluster analysis was performed using the unweighted pair-group method with average linkages (UPGMA). Profile similarities of the same isolate analyzed in separate experiments and compared in different gels ranged from 98% to 100%.

2.3. Identification of integrase genes and analysis of variable regions of class 1 and class 2 integrons

Template genomic DNA was provided as boiled lysates or isolated with Genomic Mini DNA extraction kit (A&A Biotechnology). Integron classes were determined by multiplex PCR. The sequences of primers targeting *intI1*, *intI2* and *intI3* integrase genes were recommended by Dillon et al. (2005). Amplification involved an initial denaturation (94 °C, 5 min) followed by 30 cycles of denaturation (94 °C, 1 min), annealing (59 °C, 1 min) and extension (72 °C, 1 min) with a final extension step (72 °C, 8 min). Negative control contained sterile deionized water instead of template DNA. Positive controls for *intI1* and *intI2* were *Enterobacter hormaechei* MPU E9 and *E. coli* MPU D1/7, respectively (Mokracka et al., in press). To exclude false positive results, the PCR products of the expected sizes (160 bp for *intI1*, 788 bp for *intI2* and 979 bp for *intI3*) were sequenced in a 3130xl Genetic Analyzer (Applied Biosystems). The amplicon sequences were compared against GenBank database by using BLASTn (Nucleotide Basic Local Alignment Search Tool).

The variable regions of the integrons were amplified with primers complementary to the 5′ and 3′ conserved regions of class 1 integron (5′-CS and 3′-CS according to Lévesque et al., 1995) and class 2 integron (Hep54 and Hep71 according to White et al., 2001). PCR amplifications were conducted as follow: initial denaturation 94 °C, 5 min, and 30 cycles of 94 °C 1 min, 55 °C 1 min, 72 °C 5 min, and final elongation 72 °C 8 min.

All PCR reactions were done in a C1000 Thermal Cycler (Bio-Rad). The products were separated in 1.5% agarose gel (Prona). Molecular weight of PCR products was determined