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Short Communication

Characterisation of class 3 integrons with oxacillinase gene cassettes in hospital sewage and sludge samples from France and Luxembourg ☆

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ABSTRACT

In this study, antibiotic resistance class 3 integrons in Gram-negative bacteria isolated from hospital sewage and sludge and their genetic contents were characterised. Two samples of hospital effluent from France and Luxembourg and one sample of sludge from a wastewater treatment plant in France were collected in 2010 and 2011. Bacteria were cultured on selective agar plates and integrons were detected in colonies by quantitative PCR. Integron gene cassette arrays and their genetic environments were analysed by next-generation sequencing. Three class 3 integron-positive isolates were detected, including *Acinetobacter johnsonii* LIM75 (French hospital effluent), *Aeromonas allosaccharophila* LIM82 (sludge) and *Citrobacter freundii* LIM86 (Luxembourg hospital effluent). The gene cassettes were all implicated in antibiotic (aminoglycoside and β-lactam) or antiseptic resistance. An oxacillinase gene cassette (bla_{OXA-10}, $bla_{OXA-368}$ or bla_{OXA-2}) was found in each integron. All of the class 3 integrons were located on small mobilisable plasmids. This study highlights the role of class 3 integrons in the dissemination of clinically relevant antibiotic resistance genes, notably oxacillinase genes, in hospital effluent.

1. Introduction

Bacterial resistance is increasing worldwide in humans, animals and the environment [1]. Integrons are genetic elements involved in the dissemination of antibiotic resistance, mainly among Gram-negative bacteria (GNB) [2]. Integrons are defined by three key elements: (i) an *intI* gene encoding an integrase; (ii) an *attI* recombination site recognised by IntI; and (iii) a Pc promoter [3]. Integrons are able to capture and express genes contained within mobile gene cassettes. Gene cassettes include a gene expressed via Pc and an *attC* recombination site. More than 130 gene cassettes involved in antibiotic resistance have been described [4]. Gene cassettes can be integrated into the integron or excised through a site-specific recombination event catalysed by IntI. Three main classes of integrons are involved in antibiotic resistance, with class 1 being the most frequent [2]. Fewer than ten class 3 integrons have been characterised in GNB of clinical (*Serratia* [5], *Klebsiella* [6–8] and *Escherichia* [9]) or environmental origin (*Enterobacter* [10] and *Delftia* [11]). Except in *Delftia*, all known class 3 integrons harbour gene cassettes encoding resistance to β-lactams (bla_{IMP}, bla_{GES}, bla_{BEL}, $bla_{OXA-256}$) and aminoglycosides [*aac(6′)-Ib*]. We recently showed that class 3 integrons were abundant in hospital effluents, but we did not characterise their gene cassette contents [12]. The aim of this study was to detect and characterise class 3 integrons in hospital sewage and sludge samples. Three novel class 3 integrons were detected, all harbouring oxacillinase gene cassettes.

2. Materials and methods

2.1. Environmental samples and isolation of class 3 integron-positive isolates

Two hospital effluent samples were collected, in June 2010 (Limoges University Hospital Center, Limoges, France) and June

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2011 (Emile Mayrisch Hospital Center, Esch-sur-Alzette, Luxembourg). In addition, an activated sludge sample was taken from the Limoges municipal wastewater treatment plant in May 2011. Briefly, 1 L of sludge was centrifuged and the resulting pellet was re-suspended in sterile water. The effluents were vacuum-filtered with a pore size of 0.45 µm and bacteria were re-suspended in sterile water. Serial dilutions were plated on Luria–Bertani (LB) (Mo Bio Laboratories, Carlsbad, CA) agar plates supplemented with vancomycin (32 mg/L) plus kanamycin (50 mg/L), cefotaxime (16 mg/L) or imipenem (8 mg/L) (antibiotics all from Sigma-Aldrich, Lyon, France). Plates were incubated at 30 °C for 48 h and isolated colonies were screened for class 1, 2 and 3 integrons by quantitative PCR (qPCR) [13].

2.2. DNA extraction and next-generation sequencing (NGS)

Total DNA was extracted using a QIAamp® DNA Mini Kit (QIAGEN, Courtaboeuf, France) and plasmid DNA was purified with a PureYield™ Plasmid Miniprep Purification System (Promega, Charbonnières-les-Bains, France). NGS was performed with Ion Proton™ technology (Thermo Fisher Scientific, Villebon sur Yvette, France) according to the manufacturer's instructions. Reads were assembled using MIRA (Mimicking Intelligent Read Assembly). Contigs were analysed using Geneious software (Biomatters, Auckland, New Zealand). Bacterial identification was based on analysis of the *gyrB*, *rpoB* and 16S RNA-encoding genes.

2.3. Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was performed by the agar diffusion method as recommended by the French Society for Microbiology. Minimum inhibitory concentrations (MICs) were determined by Etest (bioMérieux, Marcy-l'Étoile, France) according to the manufacturer's recommendations.

2.4. Mating and transformation experiments

Plasmid DNA was electroporated into *Escherichia coli* recipient strain DH5α (Table 1), with selection on LB agar plates containing ampicillin (100 mg/L) (Sigma-Aldrich) or kanamycin (25 mg/L). Mating experiments were also conducted in LB broth with native class 3 integron-positive isolates and *E. coli* MFD*pir*::pULIn3-9 as donors (Table 1); *E. coli* DH5α was used as the recipient strain.

3. Results and discussion

A qPCR signal for a class 3 integron was detected in three isolates, including *Acinetobacter johnsonii* LIM75 (from French hospital effluent), *Aeromonas allosaccharophila* LIM82 (from French sludge) and *Citrobacter freundii* LIM86 (from Luxembourg hospital effluent). LIM86 also harboured a class 1 integron. NGS was used to characterise the gene cassette content and genetic environment of the integrons. The three class 3 integrons were designated In3-6 (from LIM75; **LN877969**), In3-7 (from LIM82; **KT736121**) and In3-9 (from LIM86; **KT984195**) (http://integrall.bio.ua.pt/).

LIM75, LIM82 and LIM86 all expressed acquired resistance to β-lactams and aminoglycosides (Table 1). All of the genes found in In3-6, In3-7 and In3-9 were implicated in antibiotic or antiseptic resistance (Fig. 1). In3-6 harboured four gene cassettes: *aac(6′)-Ib* encoding resistance to tobramycin and amikacin; bla_{OXA-10} encoding resistance to penicillins; a new variant of *aadA1* (one amino acid substitution, Gly78Arg) encoding resistance to streptomycin and spectinomycin; and a *qacE2*-like gene with one amino acid substitution (Ala8Val), implicated in resistance to quaternary ammonium compounds. Two gene cassettes were detected in In3-7: a novel bla_{OXA} variant $bla_{OXA-368}$ (http://www.lahey.org/Studies/) and an *aac(6′)-Ib* variant [*aac(6′)-Ib′*] encoding resistance to tobramycin and gentamicin already described in In3-5 [10]. The $bla_{OXA-368}$ gene is very close to bla_{OXA-74} (one amino acid substitution, Gly149Asp), as previously described in a class 1 integron (**AJ854182**). In3-9 also contained two gene cassettes: bla_{OXA-2} previously found in several class 1 integrons [4] and the extended-spectrum β-lactamase (ESBL)-encoding gene bla_{GES-1} previously found in In3-2 [6]. Interestingly, an oxacillinase gene cassette was present in each of the three class 3 integrons (bla_{OXA-10}, $bla_{OXA-368}$ or bla_{OXA-2}). Only one other oxacillinase gene cassette ($bla_{OXA-256}$) has previously been described in a class 3 integron (In3-5) [10]. Several other reported class 3 integrons contained only the first 32 bp of bla_{OXA-10} [6,7,9]. bla_{OXA} genes are widely distributed in GNB and have frequently been reported as gene cassettes in class 1 integrons retrieved from clinical and environmental

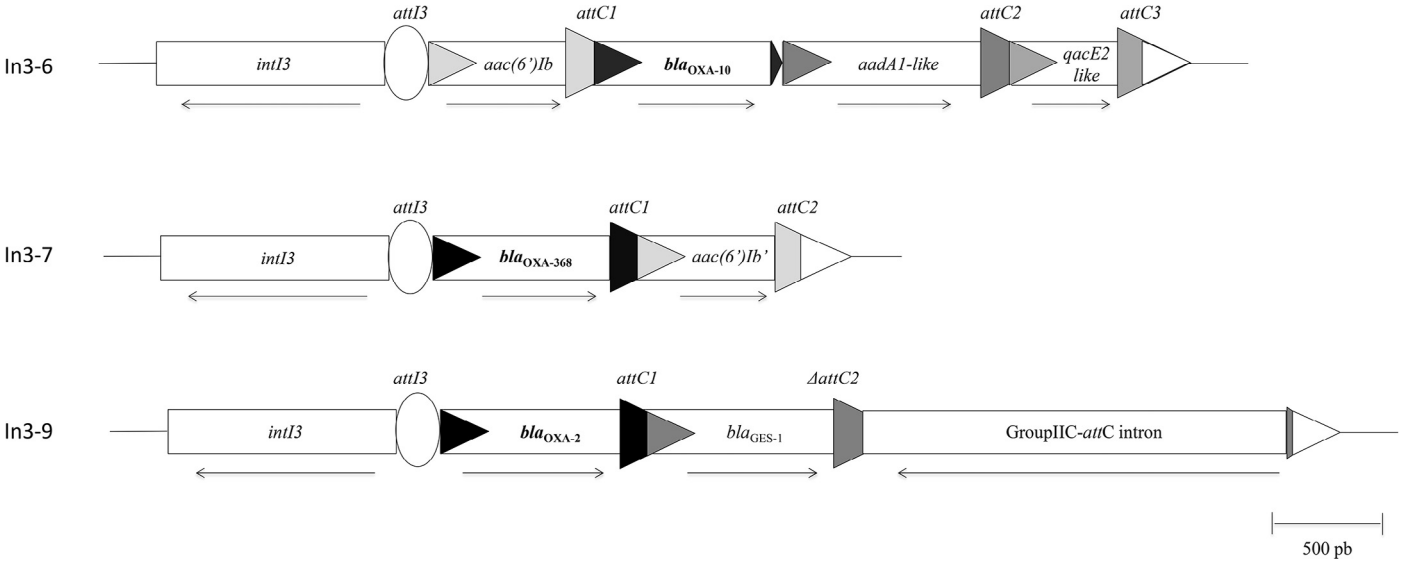

Fig. 1. Physical map of class 3 integrons In3-6, In3-7 and In3-9. Arrows represent the coding sequences of the integrase gene and gene cassettes. The recombination sites *attI3*, *attC1*, *attC2*, *ΔattC2* and *attC3* are represented by triangles; the Group IIC-*attC* intron is represented by a rectangle. *intI3*, integrase gene; bla_{OXA-10}, $bla_{OXA-368}$, bla_{OXA-2} and bla_{GES-1}, β-lactam resistance genes; *aac(6′)-Ib*, *aac(6′)-Ib′* and *aadA1*, aminoglycoside resistance genes; and *qacE2*, quaternary ammonium compound resistance gene.