Universidade de Aveiro Departamento de Biologia
2011

Alexandra Sofia Trindade Rodrigues Silva Moura

Diversidade molecular e transferência de integrões de águas residuais

Molecular diversity and transferability of integrons from wastewaters

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Biologia, realizada sob a orientação científica do Professor Doutor António Carlos Matias Correia, Professor Catedrático do Departamento de Biologia da Universidade de Aveiro.

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o júri

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MUITO OBRIGADA A TODOS!!!!

palavras-chave elementos geneticamente móveis, integrões, cassetes de genes, comunidades bacterianas, águas residuais.

Resumo

Na evolução bacteriana, a capacidade de explorar novos ambientes e de responder a diferentes pressões selectivas deve-se principalmente à aquisição de novos genes por transferência horizontal. Integrões são elementos genéticos bacterianos que constituem sistemas naturais de captura e expressão de cassetes de genes, sendo um dos principais mecanismos bacterianos envolvidos na aquisição de resistências a antibióticos. Estudos recentes suportam a hipótese de que os ambientes naturais constituem importantes reservatórios de integrões e cassetes de genes. Uma vez que as águas residuais são descarregadas em receptores naturais, torna-se fundamental conhecer a presença e dispersão de integrões nestes ambientes, assim como a sua associação a outros elementos genéticos móveis e a genes de resistências a antibióticos.

Neste trabalho, pretendeu-se avaliar a prevalência e diversidade de integrões em águas residuais de origem animal e doméstica, bem como a sua associação a plasmídeos conjugativos, usando metodologias dependentes e independentes do cultivo de microrganismos em laboratório.

Os resultados obtidos sustentam assim a hipótese de que ambientes particularmente ricos em matéria orgânica, como é o caso das águas residuais, constituem ambientes propícios à presença de integrões e à ocorrência de transferência horizontal de genes de resistência a antibióticos, embora a sua prevalência e diversidade seja influenciada pelo tipo de efluente em questão. A presença de integrões em estações de tratamento de águas residuais, e em especial nos efluentes tratados, constitui assim um factor preocupante, uma vez que tal contribui para a sua disseminação e dispersão por outros ecossistemas aquáticos, nomeadamente rios e mares.

Os métodos utilizados permitiram também detectar uma elevada diversidade de cassetes de genes associadas a integrões, sendo possível que algumas dessas sequências codifiquem para proteínas que desempenhem um importante papel na adaptação bacteriana às intensas pressões selectivas características deste tipo de ambientes.

Assim, é possível concluir que as comunidades bacterianas presentes em águas residuais reúnem diferentes tipos de elementos geneticamente móveis que desempenham um importante papel não só na adaptação bacteriana, mas também na disseminação de determinantes genéticos de resistência para ambientes naturais. Adicionalmente, a presença de potenciais proteínas com possíveis aplicações biotecnológicas reforça a importância das águas residuais como fontes de diversidade funcional.

Este trabalho incluiu também a criação e implementação da base de dados INTEGRALL, desenvolvida com o intuito de congregar informação acerca de integrões e de uniformizar a nomenclatura de cassetes de genes.

keywords mobile genetic elements, integrons, gene cassettes, bacterial communities, wastewater environments.

abstract

Integrons are genetic systems that enable bacteria to acquire and excise gene cassettes by site-specific recombination. These structures are often associated with plasmids and transposons playing an important role in horizontal gene transfer.

In this work the diversity of integrons in wastewater environments has been assessed. Specifically, the prevalence of integrons, diversity of encoding gene cassettes and their association with broad-host range plasmids was investigated using culture-dependent and independent methodologies.

Results obtained support the hypothesis that wastewater environments constitute hotspots for the presence of integrons and the occurrence of horizontal gene transfer, although its prevalence is affected by the type of effluent. The presence of integrons in wastewater treatment plants and, in particular in treated effluents, constitutes a matter of concern regarding their dissemination and spreading to other aquatic systems, such as rivers and seas.

The methodologies here applied also allowed the detection of a highly diverse reservoir of integron-borne gene cassettes in wastewater environments encoding putative proteins that may have an important role in bacterial adaptation to intensive selective pressures characteristic of these environments.

In conclusion, this study shows that wastewater environments promote the development of bacterial communities that support and bring together different types of molecular elements that in association are the main players in the dissemination of antibiotic resistance determinants to natural environments and in bacterial adaptation. In addition, the presence of putative proteins with potential biotechnological applications justifies further work in gene cassette characterization and stresses the importance of wastewaters gene cassette reservoirs as sources of functionally diverse proteins.

This work also included the implementation of INTEGRALL database, an online platform developed to congregate information on integrons and standardize gene cassette nomenclature.

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List of Publications

This thesis includes results published in the papers listed below:

Moura A., Henriques I., Ribeiro R., Correia A. (2007) Prevalence and characterization of integrons from bacteria isolated from a slaughterhouse wastewater treatment plant. *Journal of Antimicrobial Chemotherapy* 60: 1243–1250.

Moura A., Soares M., Pereira C., Leitão N., Henriques I., Correia A. (2009) INTEGRALL: a database and search engine for integrons, integrases and gene cassettes. *Bioinformatics* 25:1096–1098.

Moura A., Henriques I., Smalla K., Correia A. (2010) Wastewater bacterial communities bring together broad-host range plasmids, integrons and a wide diversity of uncharacterized gene cassettes. *Research in Microbiology* 161:58-66.

Moura A., Pereira C., Henriques I., Correia A. (2011) Novel integrons and gene cassettes in antibiotic-resistant bacteria isolated from urban wastewaters (*submitted for publication*).

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CHAPTER 1

Introduction

1.1 Horizontal gene transfer and microbial evolution

Microorganisms display a remarkable diversity of cellular structures, morphologies, lifestyles and metabolic properties, giving them the ability to exploit new environments and to respond to new selective pressures (Davison, 1999).

Several mechanisms may be responsible for such differences among microbial species (Ochman *et al.*, 2000). Those include genetic rearrangements and the accumulation of point mutations that may lead to the modification, inactivation or differential regulation of existing genes, contributing to the diversity of microbial genomes (Thomas & Nielsen, 2005). However, it has been recognised that gene transfer among different organisms, called horizontal gene transfer (HGT), may have more profound effects on genome evolution rather than the sequential modification of gene functions by accumulation of point mutations (Davison, 1999).

The flow of genetic information through HGT has been found to occur in Archaea, Bacteria and Eukarya and can overcome species and even domain boundaries (Syvanen & Kado, 2002). Studies on the extent of HGT at the domain level have been performed using *in silico* analysis of approximately 8000 protein families (Choi & Kim, 2007). It has been estimated that more than 50% of Archaea and 30-50% of Bacteria may possess one or more protein domains acquired by HGT (Choi & Kim, 2007). Among Bacteria and at a lower taxonomical level, earlier studies based on whole genome analysis have also estimated that nearly 20% of genes of *Escherichia coli* have been acquired by HGT (Lawrence & Ochman, 1998). HGT is therefore considered a major driving force in shaping genome evolution (Cordero & Hogeweg, 2009).

There are three distinct mechanisms by which horizontal gene transfer may occur: a) transformation, in which a cell takes up free DNA molecules from the surrounding medium, b) conjugation, which involves the direct transfer of DNA from one cell to another, and c) transduction, in which the transfer is mediated by bacteriophages (for a review, see Thomas & Nielsen, 2005).

On the last decade, genome sequencing accomplishments have shown that besides core genes that encode essential metabolic functions, prokaryotic genomes also harbour a variable number of accessory genes acquired by HGT (**Figure 1.1**; Schmidt & Hensel, 2004).

The majority of the genes of the *core gene pool* (**Figure 1.1**) encodes proteins that play roles in basic cellular functions (such as DNA replication and essential metabolic functions) and exhibit homogeneous G+C content and codon usage (Hacker & Carniel, 2001).

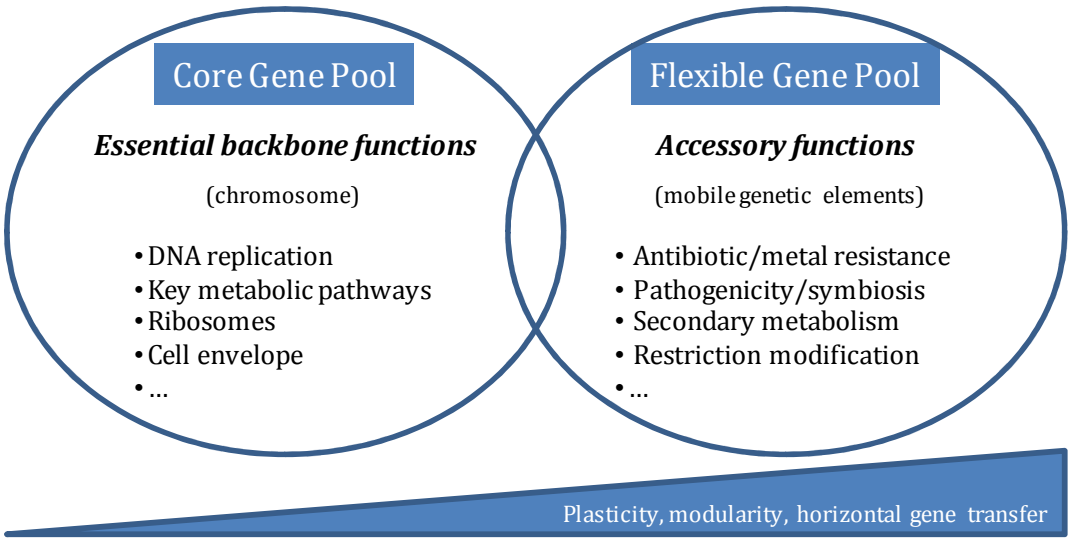

Figure 1.1 Model of the DNA pools in prokaryotic genomes, comprising the core genome and the flexible genome (based on Heuer *et al.*, 2008 and Hacker & Carniel, 2001).

The *flexible gene pool* comprises genes often located in mobile genetic elements (MGEs), such as virus, plasmids, transposons, genomic islands and integrons that serve as vehicle for the spread of adaptive traits (**Figure 1.1**). Elements of the flexible gene pool often have different G+C content and codon usage, and encode additional functions that are not essential to the cell, but advantageous in certain conditions (Hacker & Carniel, 2001).

MGEs are usually expendable and thus prone to be lost, unless they favour fitness advantages to host cells. To be maintained in their host cell for an extended period of time, MGEs must contribute with functions that provide selective advantage to host cells, have an effective system to ensure accurate segregation at cell division (in case of extrachromosomal elements) or behave as a parasitic component capable of infectious HGT (Bahl *et al.*, 2009b).

Continuum HGT events may lead to the stable inheritance of MGEs into a new host, resulting in novel genetic combinations and acting as a driving force in genomic innovation, thus compensating asexual reproduction (Smets & Barkay, 2005).

Due to the emergence of pathogenic strains associated with the acquisition of numerous virulence factors and resistant determinants, HGT and MGEs have been receiving increased attention. In particular, gene acquisition mediated by integrons is considered an important pathway for facilitating the spread of antibiotic resistance genes across bacteria.

1.2 The integron/gene-cassette system

1.2.1 The role of integrons for bacterial evolution

Integrons are genetic elements that contain the determinants of a site-specific recombination system by means of which they facilitate the integration, excision and rearrangements of discrete genetic elements, known as gene cassettes (Stokes & Hall, 1989; Cambray *et al.*, 2010). Integrons are active players in bacterial adaptation, playing a major role in the acquisition and expression of new genetic determinants (Boucher *et al.*, 2007; Guerin *et al.*, 2009).

Integrons were first identified in 1987 in a clinical context (Stokes & Hall, 1989). Common regions were noted upstream and downstream of various antibiotic resistance genes in different genomic locations, suggesting that, like transposons, these structures were mobile. However, these elements differed from transposons in two distinct features. On the one hand, unlike transposons, which are flanked by direct or indirect repeat sequences, the regions surrounding the antibiotic resistance genes in the new elements were not repeats. On the other hand, the elements contained a site-specific integrase gene of the same family as those found in phage lambda but lacked many gene products associated with transposition. Due to these differences, these elements were not grouped with transposons and were named integrons (Stokes & Hall, 1989).

1.2.2 The integron platform - structure and transferability

The basic structure of an integron consists of a platform containing four elements: an integrase gene (*intI*), its associated promoter (P_{int}), one or two promoters responsible for gene cassette expression (Pc1-Pc2), and a recombination site (*attI*) (**Figure 1.2**; Cambray *et al.*, 2010). These features are found in a module of approximately 1.4 kb, known as the 5'-conserved segment (5'-CS).

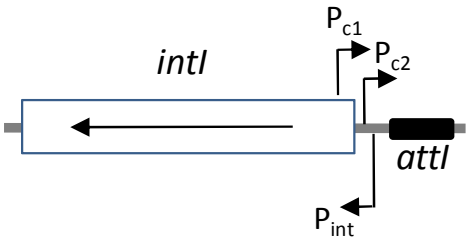

Figure 1.2 The structure of the integron platform: the *intI* gene that codes for integron integrase; the Pint promoter, responsible for expression of the integrase gene; the *attI* recombination site; the Pc1 promoter responsible for the expression of the gene cassette array; the second promoter Pc2 (if present) that generally enhances the expression of gene cassette arrays (based on Jové *et al.*, 2010).

The *intI* gene encodes an integron integrase (IntI) that belongs to tyrosine recombinase family. This family includes proteins that recombine DNA duplexes by executing two consecutive strand breakage and rejoining steps and a topoisomerization of the reactants. Enzymes belonging to the tyrosine recombinase family possess three invariant residues: a His–X–X–Arg cluster and a Tyr residue (Esposito & Scocca, 1997). The first member of this family, the well-studied lambda integrase protein (Int), promotes integration and excision of the phage genome into the host's genome; other family members function in the maintenance of plasmid copy number, on the elimination of dimers from replicated chromosomes and in the alteration of cell-surface components, as well as in the life cycle of temperate phages. Integron integrases form a subclade in this family by the presence of a specific additional protein segment required for their activity (Bouvier *et al.*, 2009).

The integron integrase catalyses the insertion and excision of gene cassettes by site-specific recombination. Recombination takes place between two recombination sites, primarily at the *attI* recombination site, located in the 5'-CS of

the integron platform, downstream the *intI* gene (**Figure 1.3**). The *attI* sites of different integron classes do not share substantial sequence identity with each other. The extent of *attI1* sequence required for maximal site activity has been determined in previous studies. The full (65-bp) *attI1* site is required for efficient recombination with *attC* (Recchia *et al.*, 1994), although some authors suggest that a smaller sequence is sufficient to support a lower level of recombination (Partridge et al., 2000).

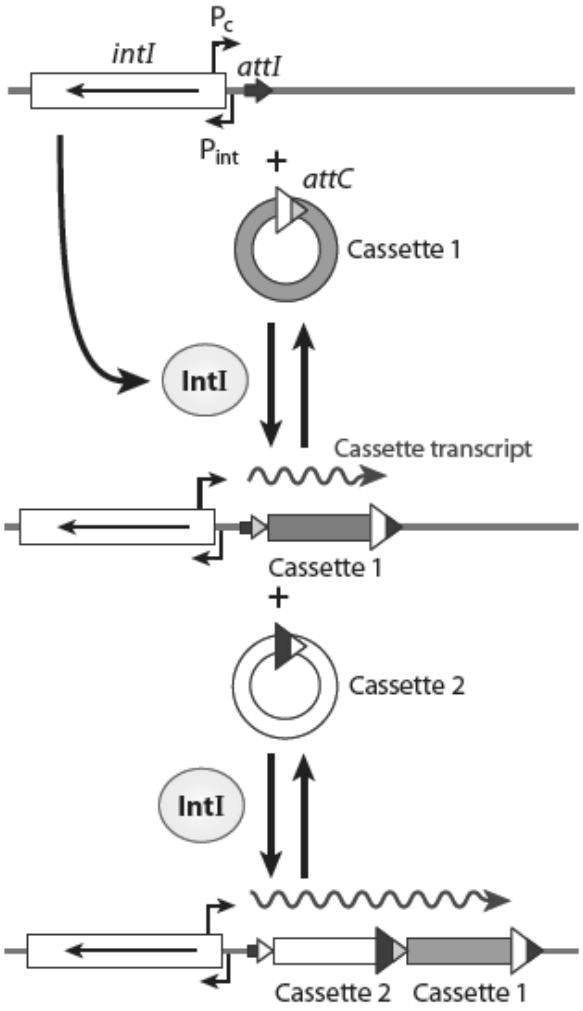

Figure 1.3 Mechanism of integron-mediated gene capture. Integrase catalyses the insertion or excision of circular gene cassettes at the *attI* site through site-specific recombination (Cambray *et al.*, 2010).

Recombination between two *attC* sites (*attC x attC* recombination) mediated by integrase may also occur, although less efficiently, resulting in gene cassette deletion through circular intermediates (Bouvier *et al.*, 2005); no evidences for recombination between two *attI* sites has been reported. Integron integrases may recognize several distinct *attC* sites as substrates, since low sequence identity is necessary for substrate identification. In contrast to other tyrosine recombinase substrates which require a common 6-8 nt core sequence, only three completely conserved nucleotides (GTT) are necessary in the substrates of integron integrases (Frumerie *et al.*, 2010).

Culture-independent studies as well as the analysis of complete sequenced genomes have demonstrated that the gene family of integrases is vast, encoding related but distinct integrases, that potentially share the same gene cassette pool (Nemergut *et al.*, 2004; Boucher *et al.*, 2007).

1.2.2.1 Classification of integrons

Integrons are frequently associated with other MGEs, such as transposons, insertion sequences and plasmids (**Figure 1.4**).

This led to the categorization of integrons in two sets: the mobile or multi-resistance integrons (MRIs), which are linked to MGEs and that may contain up to eight gene cassettes encoding antibiotic resistance, and the sedentary integrons (SIs, also referred to as chromosomal integrons or super-integrons), which are ancient static elements that may contain hundreds of gene cassettes of mostly unknown function.

At present, five classes of MRIs have been identified according to the IntI sequence, sharing 40–59% of amino acid sequence similarity (**Table 1.1**).

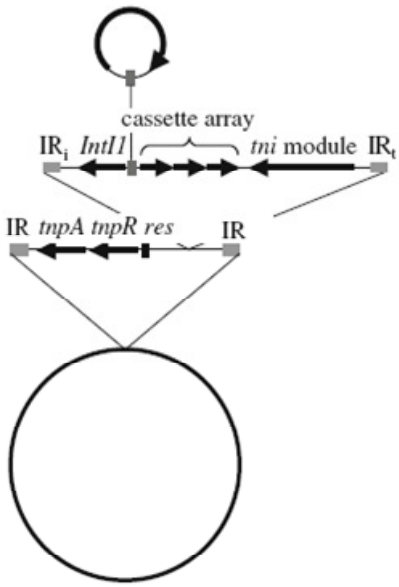

Figure 1.4 The hierarchical composition of mobile genetic elements. Gene cassettes may be incorporated into integrons. Integron flanked by inverted repeats (IR) may be inserted into a composite transposon, which in turn may be inserted into self-transmissible plasmid (Bahl *et al.*, 2009b).

It is believed that integrons may have already been present or even widely spread in bacteria prior to the introduction of antibiotics in medicine and subsequently moved into human pathogens. The diversity of backbone structures present in integrons also suggests an evolutionary history for these elements longer than the 50 years of extensive antibiotic use. Several evidences suggest that the emergence of MRIs that mostly contain resistance cassettes might have occurred through the association of chromosomal integrons with mobile elements. In comparison to MRIs, SIs tend to contain larger cassette arrays, have greater homogeny in *attC* sites and contain mostly gene cassettes coding for proteins with unknown function (Coleman & Holmes, 2005).

Table 1.1 Amino acid identity among integrases belonging to classes 1 to 5 (Jové, 2010).

Integrase	IntI1	IntI2	IntI3	IntI4	IntI5
IntI1	100%				
IntI2	46%	100%			
IntI3	59%	47%	100%		
IntI4	40%	54%	44%	100%	
IntI5	41%	44%	44%	40%	100%

The general features and recent advances in the understanding of integron structure, diversity, evolution and transferability are summarized below, focusing on MRIs belonging to class 1 to 3. The other two classes of MRIs, class 4 and class 5, possess only a few representatives. Both classes have been identified as involved in trimethoprim resistance in *Vibrio* species. The class 4 integron is usually embedded in an integrative and conjugative element found in *Vibrio cholerae* (Hochhut *et al.*, 2001) and the class 5 integron has been found located in a plasmid-borne composed transposon in *Vibrio salmonicida* (GenBank accession no. AF179595).

1.2.2.2 Class 1 integrons

Class 1 integrons are the most extensively studied class of integrons due to their implication in hospital-acquired infections and association with multi-resistance phenotypes to antibiotics (Leverstein-van Hall *et al.*, 2002). Their occurrence has been reported mostly in Gram-negative bacteria, mainly Proteobacteria, and in a few Gram-positive (**Chapter 7**; Nandi *et al.*, 2004).

Class 1 integrons are frequently associated with conjugative plasmids belonging to narrow- (e.g. IncFI, IncFII, and IncL/M) and broad-host-range incompatibility groups (e.g. IncP, IncQ, IncW and IncN), usually embedded within Tn*21*-related transposons (for a review see Liebert *et al.*, 1999). The extensive dissemination of Tn*21*, and consequently of class 1 integrons, is thought to result from the association of the integron with the *mer* operon (conferring

resistance to mercury) when the loads of antibiotics and heavy metals in clinical, agricultural and industrial practices were rapidly increasing (Liebert *et al.,* 1999).

Class 1 integrons derivatives from Tn*21* often contains a complete or partial 3′-conserved segment (3′-CS), in addition to the 5′-conserved segment referred before (**Figure 1.5**). The 3′-CS usually consists of a truncated version of the gene cassette *qacE* (*qacEdelta1*) which encodes low resistance to quaternary ammonium compounds, such as antiseptics and disinfectants, a *sul1* gene that encodes a sulphonamide resistance dihydropteroate synthetase and an open reading frame (termed ORF5) which product has some similarity to puromycin acetyltransferase, involved in antibiotic resistance. Occasionally, an additional ORF (ORF6) of unknown function may also be present. The truncation of *qacEdelta1* may have evolved due to the insertion of a *sul1* at the 3′ end of the gene, thus removing the *attC* element of *qacE* gene cassette and some of the coding sequence.

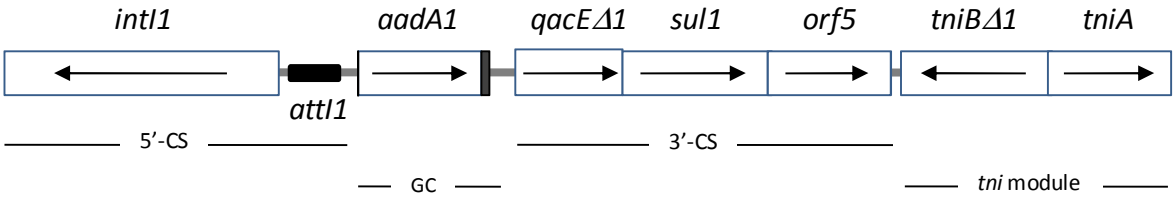

Figure 1.5 Structure of Tn*21*-borne In2 integron (accession no. AF071413), showing the typical 3′-conserved segment (adapted from Liebert *et al.,* 1999). Black boxes represent recombination sites (*attI* and *attC*); CS, conserved segment; GC, gene cassette.

It has been hypothesised that Tn*402* (also termed Tn*5090*) is an ancestor of Tn*21*-embebedd class 1 integrons, since Tn*402*-derivatives lack the 3′-CS (Gillings *et al.*, 2008). The appearance and establishment of the 3′-conserved segment is thought to be a relatively recent event, resulting from the introduction of sulphonamides in clinical practice that led to the fixation of the *sul* gene. Tn*402* found in the IncP-1β plasmid R751, is an active transposon that contains a

transposition gene module (*tni* module) of four genes (*tniA*, *tniB*, *tniQ* and *tniC* (also referred as *tniR*)).

Several class 1 integrons lacking 3'-CS have been reported containing complete *tni* modules or remnants of the *tni* set, which render it a defective transposon. However, integrons possessing a 3'-CS are likely to have selective advantage due to the presence of *sul1* and are clearly more reported than those associated to Tn*402*. Nevertheless, this may be due to experimental bias, since most of studies rely exclusively on the use of primers targeting 5'CS and 3'CS regions for integron characterization.

1.2.2.3 Class 2 integrons

Class 2 integrons have been mostly associated with conjugative IncF, IncL/M, IncN and IncP-1α plasmids in *Escherichia coli* and *Shigella* spp. (Partridge *et al.*, 2009). Class 2 integrons are most often found within transposon Tn*7* and its relatives (**Figure 1.6**). Tn*7* is an active transposon that contains a transposition module of five *tns* genes (A, B, C, D and E) and preferentially inserts into a unique site within bacterial chromosomes (Craig, 1991).

Class 2 integrons usually possess a defective integrase, due to the presence of a premature in-frame stop codon in *intI2* gene (Hansson *et al.*, 2002). However, recent studies have reported the presence of *intI2* genes with a glutamine CAA codon instead of the TAA stop codon in *Providencia stuartii* and *E. coli* isolated from beef cattle sources and the human urinary tract, respectively (Barlow *et al.*, 2009; Márquez *et al.*, 2008). The activity of IntI2 containing the glutamine codon has been further experimentally confirmed in a human pathogenic *E. coli*, mediating a urinary tract infection in Uruguay (Márquez *et al.*, 2008).

Due to the presence of the defective integrase, the diversity of gene cassette arrays associated to class 2 integrons have been limited to nearly 10 (**Chapter 7**), most of them consisting of different rearrangements of the genes *sat2, aadA1* and

dfrA1, encoding resistance to streptothricin, aminoglycosides and trimethoprim, respectively.

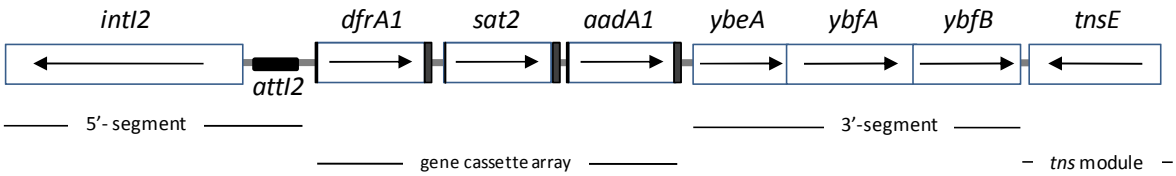

Figure 1.6 Structure of a Tn*7*-borne class 2 integron (accession no. NC_002525), showing the 3'-segment with *ybeA*, *ybfA* and *ybfB* genes (adapted from Liebert *et al.*, 1999). Black boxes represent recombination sites (*attI2* and *attC*); the *tns(D,C,B,A)* located downstream *tnsE* were omitted.

Recently, Rodriguéz-Mingela and co-workers (2009) have evaluated the *intI2* distribution beyond the usual *Enterobacteriaceae* hosts by G+C fractionation of total DNA extracted from manured soil. Results obtained suggested that *Firmicutes* (*Clostridium* and *Bacillus*) and *Bacteroidetes* (*Chitinophaga* and *Sphingobacterium*) may also be important hosts of class 2 integrons.

Furthermore, the frequent detection of class 2 integrons in manured soil (Rodriguéz-Mingela *et al.*, 2009), food production animals (Barlow *et al.*, 2009) as well as slaughterhouse wastewaters (**Chapter 4**) suggest that bacteria inhabiting animal guts, in particular pigs, may be important reservoirs of class 2 integrons.

1.2.2.4 Class 3 integrons

Class 3 integrons appear to be rare in both clinical and natural environments and so far only few representatives of this class have been found.

The first class 3 integron was identified in a clinical *Serratia marcescens* in Japan (Arakawa *et al.*, 1995), associated with Tn*402*-like transposition functions. In Portugal, a clinical *Klebsiella pneumoniae* strain carrying a similar structure was also identified (Correia *et al.*, 2003). Environmental class 3 integrons have been identified in the chromosome of two *Delftia* species isolated from wastewaters, namely a *Delftia acidovorans* from United States and *Delftia tsuruhatensis* from Canada, lacking Tn*402*-like transposition functions (Xu *et al.*, 2007). More recently, the location of a class 3 integron has been established in an IncQ broad-host-range plasmid in a clinical *E. coli* strain from France (**Figure 1.7**; Poirel *et al.*, 2010).

Five different *intI3*-associated cassette arrays have been reported, three of them include known resistance genes to β-lactams and aminoglycosides.

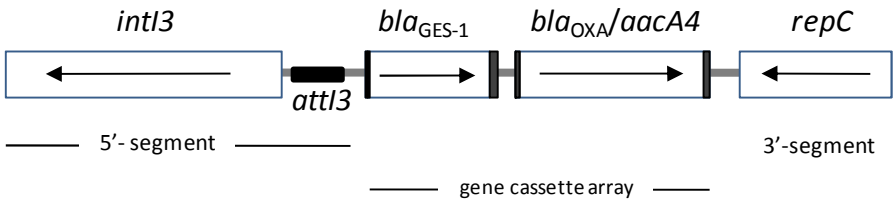

Figure 1.7 Structure of the class 3 integron found in the IncQ plamid from *E.coli* (accession no. NC_014356). Black boxes represent recombination sites (*attI3* and *attC*).

1.2.3 Structure and expression of gene cassettes

Integrons act as natural cloning and expression devices that incorporate gene cassettes and convert them to functional genes. The mobile gene cassettes are usually promoterless open reading frames (ORFs) with a gene cassette-associated recombination site (*attC*, formerly termed as 59 base-element or 59-be) recognized by an IntI integrase (Stokes *et al.*, 1997).

Gene cassettes may exist freely as circular molecules or incorporated into the integron platform in its linear form (**Figure 1.8**). Since the large majority of gene cassettes do not possess a promoter, they are dependent on the promoter(s) located in the integron and thus their expression can only occur once integrated in the integron platform in the correct orientation. This mechanism allows organisms to express acquired genes independently of foreign promoters, and therefore facilitates gene transfer among highly divergent groups of bacteria.

Although they constitute few exceptions, gene cassettes that contain their own promoter have been reported, as in the case of *qac*, *cmlA* and *ereA* gene cassettes (Guerineau *et al.*, 1990; Bissonnette *et al.*, 1991, Biskri & Mazel, 2003).

attC recombination sites can greatly vary in sequence and in length, ranging from 55 to 141 bp (for a review, see Partridge *et al.*, 2009). Each *attC* possesses short regions designated 1L and 2L at the 5´boundary (left hand simple site, LH) and 1R and 2R at the 3' boundary (right hand simple site, RH) (**Figure 1.8**). The core sites on LH and RH sites exhibit complementarity to generate a stem-loop structure (Stokes *et al.*, 1997). This secondary structure acts as structural recognition determinant for recombination that occurs between boxes 1R and the bottom strand (Jacquier *et al.*, 2009). Additionally, the correct orientation constrain imposed by the gene cassette promoter (Pc) is achieved only if *attC* is located downstream of the gene (Stokes *et al.*, 1997).

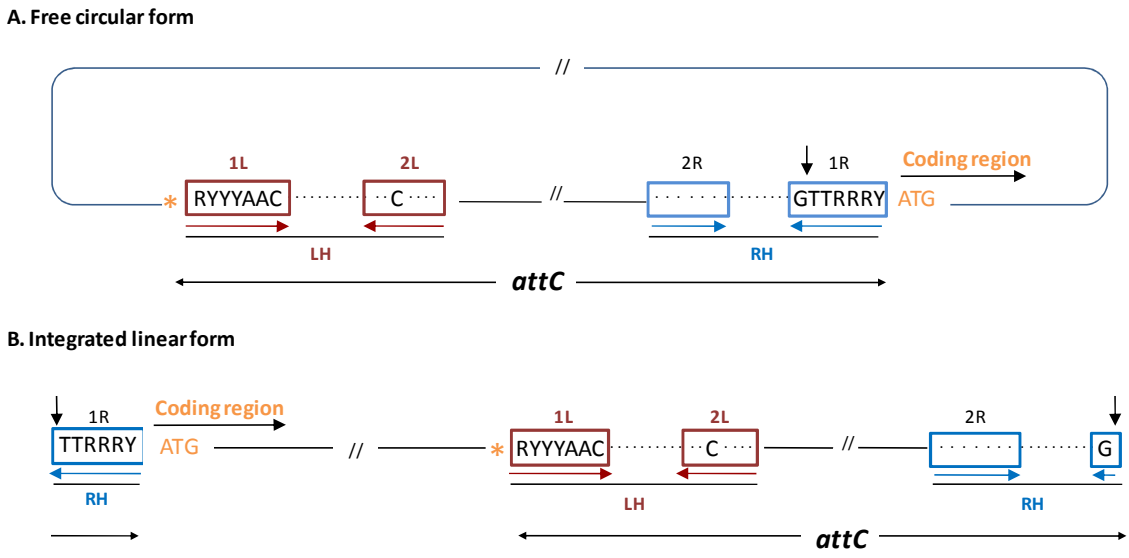

Figure 1.8 Schematic presentation of a gene cassette in its circular (A) and linear (B) forms. Gene coding region is marked by start (ATG) and stop (*) codons; core sites (sequences of 7 bp) are boxed and their relative orientations are indicated with arrows; left hand (LH, purple boxes) and right hand (RH, blue boxes) simple sites consist of pairs of integrase binding sites (1L and 2L; 2R and 1R, respectively); the vertical arrow indicates the recombination point, located between G and TT at the binding site 1R (based on Hall, 2002).

When multiple cassettes are present at the integron, the distance between the Pc and a given gene cassette may affect its level of expression: the closer the gene to Pc, the higher its expression level. This difference is thought to result from premature transcription termination at the 3´ boundaries of the cassettes, with the *attC* sites hypothetically acting as Rho-independent transcription terminators (Collins & Hall, 1995; Jacquier *et al.*, 2009). Also the extreme length of the mRNA transcript required makes unlikely the transcription of large arrays (Michael & Labatte, 2010). However, in large arrays such as the 116-cassette array of *Vibrio* sp. DAT722, the expression of distant gene cassettes has been proven to rely on the presence of different intra-array promoters (Michael & Labatte, 2010).

In class 1 integrons, several configurations of Pc1-Pc2 promoters have been identified, differing in prevalence and expression strength (Jové *et al.*, 2010;

Chapter 5). Pc1 is located within the integrase gene and, as a consequence, any nucleotide substitutions at promoter level may affect IntI1 activity. The presence of the second promoter (Pc2) may in some cases compensate a weak Pc1 without changing the activity of the integrase (Jové *et al.*, 2010). Pc2 is located within the *attI* site and is created by the insertion of three G residues that optimizes the 17 nt spacing between -35 and -10 hexamers, usually enhancing gene cassette expression (Jové *et al.*, 2010).

Recent findings also suggest that gene cassette expression can also be determined during translation, since *attC* sites can prevent the ribosome progression along the mRNA by forming a stem-loop structure at mRNA (Jacquier *et al.*, 2009).

Despite the importance of integrons in the acquisition and spread of antibiotic resistance determinants, little is known about the dynamics and regulatory control of integrase activity. A recent report by Guerin and co-workers (2009) has implicated integron activity as part of bacterial SOS response mechanisms. The alignment of the upstream region of several chromosomal and mobile integrase genes allowed the identification of a conserved LexA-binding motif overlapping the putative promoter regions (Guerin *et al.*, 2009). LexA is the transcriptional repressor governing the SOS response, a widespread regulatory network aimed at addressing DNA damage by repairing or bypassing lesions (Guerin *et al.*, 2009). Derepression of SOS genes results from the autocatalytic cleavage of LexA, a process induced by single-stranded DNA and mediated by RecA (**Figure 1.9**). The SOS response has strong links with bacterial adaptation and has been implicated in clinically relevant phenotypes, such as dissemination of virulence factors. In addition, the recombination requires the production of single-strand DNA which formation can be induced by an antibiotic stress response. This creates the possibility to use folded ssDNA as sensor of environmental stress triggering bacterial adaptation, as proposed by Loot *et al.* (2010).

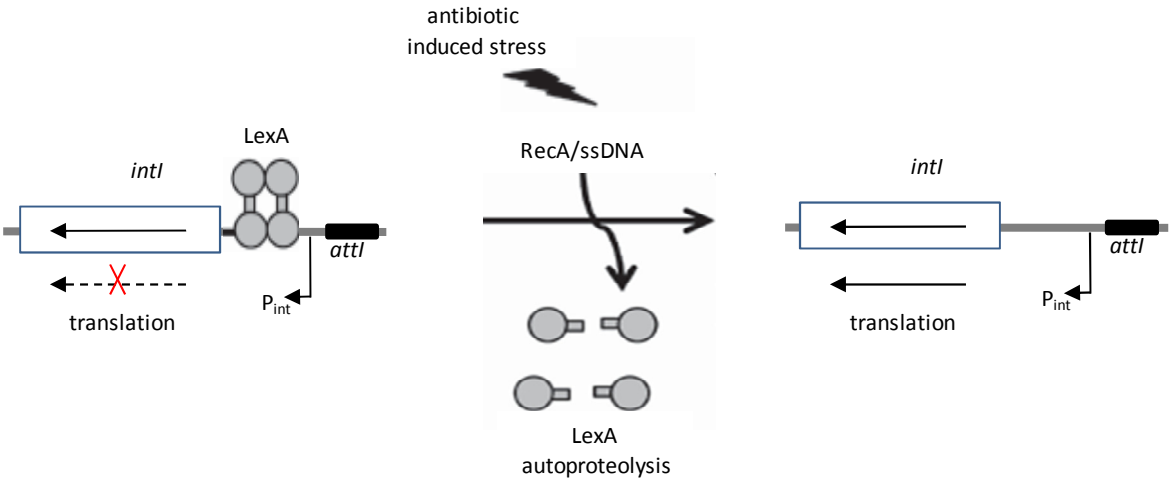

Figure 1.9 Model of regulation of integrase gene expression. In the uninduced state, the LexA protein is bound to its site at the promoter region of the integrase gene, blocking the expression of *intI*. The induction of the SOS response results in the production of ssDNA that binds to RecA, activating its co-protease activity. The interaction between LexA and the nucleoprotein filament RecA/ssDNA results in autoproteolytic cleavage of LexA, leading to *intI* derepression and subsequently to its translation (based on Da Ré *et al.*, 2009).

1.2.4 The gene cassette reservoir – origin and diversity

The lack of promoters in gene cassette (with a few exceptions) and the fact that, overall, they generally contain reduced noncoding sequence have led to the hypothesis that the formation of gene cassettes is mediated by reverse transcription of mRNA molecules (Hall, 2002). In this hypothesis, the *attC* site is thought to be added either prior to or following the reverse transcription reaction, which is consistent with the observations that some structural genes are homologous to gene cassette open reading frames (Hall, 2002).

Another specific gene cassette synthesis theory has been proposed based on the observation of group II introns inserted before *attC* sites (Leon & Roy, 2009). Bacterial group II introns are genetic elements that are both catalytic RNA

molecules and retroelements. Gene cassette synthesis is suggested to occur via a two-step mechanism in which an intron inserts before an *attC* site and a second intron inserts downstream of a structural gene. Recombination between the two introns would subsequently bring the structural gene and the *attC* site together with the intron between them. Splicing of the intron followed by reverse transcription would result in a newly formed gene cassette (Leon & Roy, 2009). Association of introns with integrons or in the genomes of integron-carrying bacteria are rare, although it has been suggested that some environmental bacteria may act as gene cassette factories (Leon & Roy, 2009).

Despite the differences among *intI* genes, each encoding related but distinct integrases, different integron classes appear to share the same gene cassette pool. Most of gene cassettes so far characterized concerning bacteria isolated in clinical environments encode determinants of resistance to antibiotics (**Table 1.2**). More than 130 different gene cassettes (based on a 98% identity cut-off) have been identified in MRIs. These include resistance genes towards β-lactams, aminoglycosides, trimethoprim, chloramphenicol, rifampicin, sulfonamides, macrolides, lincosamides, quinolones and quaternary ammonium compounds (**Chapter 7**; Partridge *et al.*, 2009). Integrons carrying up to nine antibiotic resistance gene cassettes in tandem have been reported (Naas *et al.*, 2001).

However, this does not necessarily reflect the entire diversity or distribution of gene cassettes, but instead may be due to experimental bias toward identifying such genes in clinical settings. Several studies have indeed identified MRIs harbouring unidentified gene cassettes that may be not related to antibiotic resistance (e.g. **Chapter 4**; Nemec *et al.*, 2004; Barlow *et al.*, 2009).

Table 1.2 Commonly found gene cassettes encoding antibiotic resistance.

Gene(s)	Product	Phenotype
aacA (synonym *aac*(6′))	aminoglycoside 6′-N-acetyltransferase	resistance to aminoglycosides, such as amikacin and tobramycin
aacC (synonym *aac*(3))	aminoglycoside 3-N-acetyltransferase	resistance to aminoglycosides, such as gentamicin, kanamycin and tobramycin
aadA (synonym *ant*(3′′))	aminoglycoside 3′′-adenyltransferase	resistance to aminoglycosides, such as streptomycin and spectinomycin
aadB (synonym *ant*(2′′))	aminoglycoside 2′′-adenyltransferase	resistance to aminoglycosides, such as kanamycin, gentamicin and tobramycin
aphA	aminoglycoside 3'-phosphotransferase	resistance to aminoglycosides, such as gentamicin, kanamycin and ribostamycin
arr	ADP-ribosyl transferase	resistance to rifampicin
blaBEL, blaGES, blaTLA, blaVEB	extended-spectrum class A β-lactamase	resistance to β-lactams, including expanded-spectrum cephalosporins and aztreonam
blaP (synomym *blaPSE, blaCARB*)	class A β-lactamase	resistance to β-lactams, including carbenicillins
blaIMP, blaVIM, blaGIM,blaDIM, blaSIM, blaTBM	class B β-lactamase (metallo-β-lactamase)	resistance to β-lactams except monobactams
blaOXA	class D β-lactamase (oxacillinase)	resistance to β-lactams including ampicillin, cefalothin, oxacillin and cloxacillin
catB	chloramphenicol acetyltransferases	resistance to chloramphenicol
cml	chloramphenicol exporter	resistance to chloramphenicol
dfr (synonym *dhfr*)	dihydrofolate reductase	resistance to trimethoprim
ereA	erythromycin esterase	resistance to erythromycin
qac	quaternary ammonium compound efflux protein	resistance to quaternary ammonium compounds
qnr	fluoroquinolone resistance protein	resistance to fluoroquinolones
sat	streptothricin acetyltransferase	resistance to streptothricin

Among the gene cassettes found in completely sequenced small chromosome of the V*ibrio cholerae*, potential functions have been proposed on the basis of relationships with known proteins but the majority of ORF remain unidentified (Rowe-Magnus *et al.*, 1999; Heidelberg *et al.*, 2000). Similarly, among gene cassettes recovered by amplification of total community DNA, few contain antibiotic resistance genes or potential resistance genes, but the majority of the gene cassettes found determines other functions or encode putative proteins with no homologues of known function, suggesting that the gene cassette pool is vast and a source of genetic diversity and novelty (e.g. **Chapter 2**; Stokes *et al.*, 2001; Elsaied *et al.*, 2007; Koenig *et al.*, 2008; Koenig *et al.*, 2009).

1.3 Ecological and health impacts of environmental reservoirs of antibiotics, integrons and gene cassettes

1.3.1 Persistence of antibiotics in the environment

The occurrence and impacts of human and veterinary pharmaceuticals in the environment are an emerging environmental concern. In particular, the persistence of antibiotic residues and their potential implications in the widespread resistance of bacterial pathogens is an important issue.

Antibiotics are widely used in both humans and animals for treating bacterial infections (therapeutic usage). In addition, antibiotics are used as feed additives and prophylactic agents in aquacultures. Antibiotics have also been extensively used as growth promoters in livestock animal production. In 2006, the European Union has completely banned the usage of antibiotics as growth promoters, but it their usage is still allowed in United States and Canada. Most active ingredients in antibiotics are only partially metabolized in the organisms and thus are excreted as a mixture of metabolites and bioactive forms (Kim & Aga, 2007), leading to the introduction of large amounts of antibiotics into the environment via a number of pathways (**Figure 1.10**).

The occurrence and persistence of antibiotics in the environment have been evaluated in several studies (e.g. Kümmerer, 2000; Golet *et al.*, 2002; Giger *et al.*, 2003; Lindberg *et al.*, 2007; Li *et al.*, 2008; Zuccato *et al.*, 2010). Although β-lactams, such as penicillins and cephalosporins, are the most prescribed antibiotics (Muller *et al.*, 2007), they tend to be less persistent in the environment, probably due to their high susceptibility to hydrolysis by β-lactamases (Li *et al.*, 2008; Le-Minh *et al.*, 2010). Tetracyclines can also persist for relatively long periods, despite being inactivated by photo-degradation (Kümmerer, 2009). Sulfonamides, fluoroquinolones and macrolides, albeit being relatively less prescribed (Muller *et al.*, 2007; Grave *et al.*, 2010), are found to be the most frequently detected antibiotics in water environments (Huang *et al.*, 2001; Golet *et al.*, 2002). Their

persistence in the environment is thought to be related to their high affinity to solids, leading to their concentration in particles (Le-Minh *et al.*, 2010).

The persistence of antibiotics in the environment create greater concerns about the potential effects on aquatic microbial communities and impacts on public health, as the high antibiotic concentrations released may exert selective pressures that favour the proliferation of antibiotic-resistant bacteria and mobile genetic elements (Baquero, 2008). At sub-inhibitory concentrations, antibiotics may also act as signalling molecules, triggering the expression of virulent factors, increasing bacterial motility and inducing the formation of biofilms (Linares *et al.*, 2006).

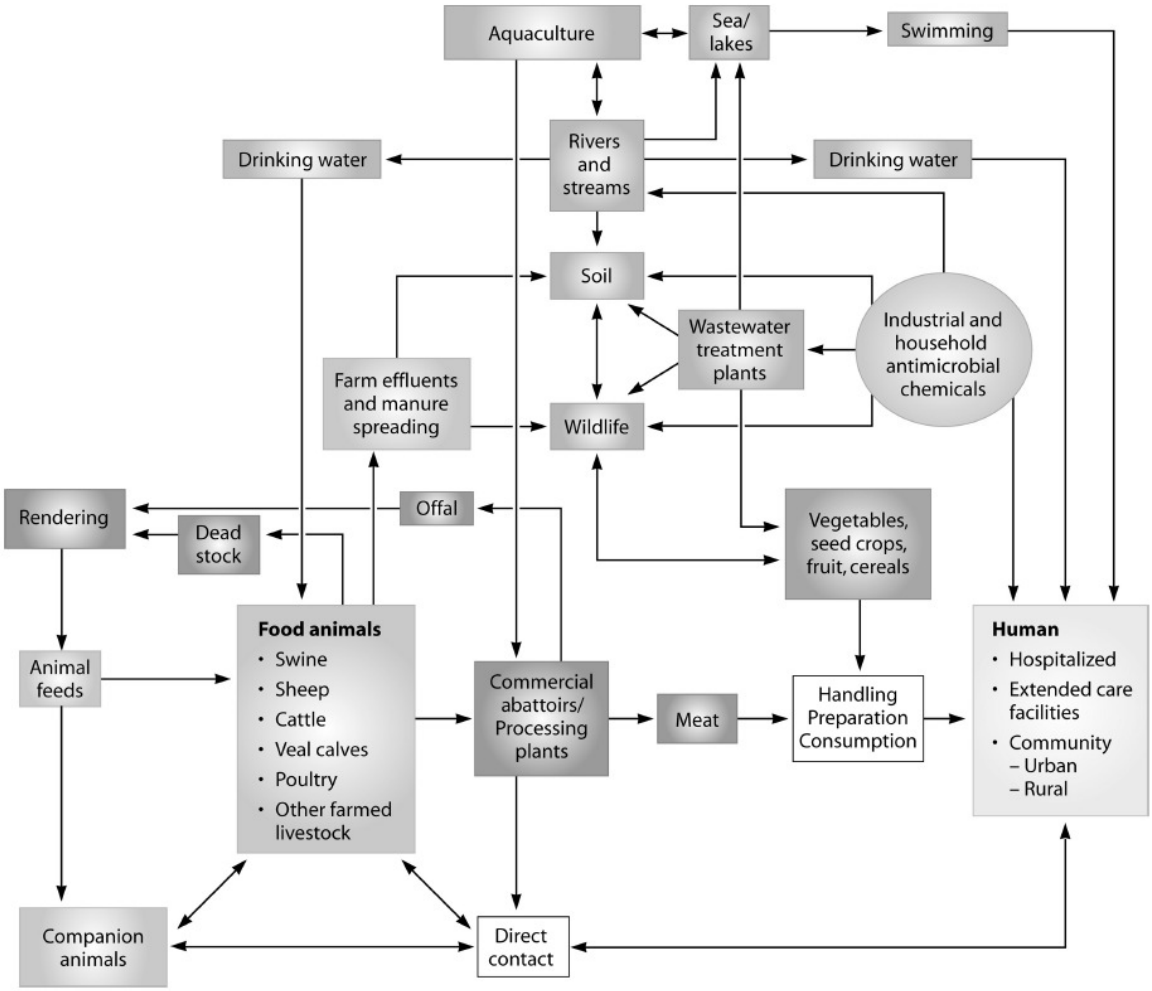

Figure 1.10 Routes of dissemination of antibiotics into the environment (Davies & Davies, 2010).

1.3.2 The environmental integron and gene cassette pool

Antibiotic-resistant bacteria pose a potential threat to human health because they limit treatment options, contributing to higher mortality levels. Antibiotic-resistant bacteria are bacteria that have acquired resistance, or are inherently resistant, to antimicrobials that would otherwise limit their growth or kill them.

The preponderant role of integrons in the capture and dissemination of antibiotic resistance genes in Gram-negative bacteria within clinical environments is now recognised. Integrons are frequently detected worldwide in clinical pathogens, such as *Acinetobacter baumannii* (Da Silva *et al.*, 2002), *E. coli* (Leverstein-van Hall *et al.*, 2002), *Shigella* sp. (Leverstein-van Hall *et al.*, 2002), *K. pneumonia* (Leverstein-van Hall *et al.*, 2002), *Pseudomonas aeruginosa* (Girlich *et al.*, 2002) and *Aeromonas hydrophila* (Libisch *et al.*, 2008).

In the last decade, studies focusing on the prevalence of integrons outside the clinical settings have emerged with increasing evidences that natural environments could serve as a source and sink of antibiotics.

The first study concerning integrons in non-clinical strains was performed by Rosser & Young (1999), who screened the presence of class 1 integrons among estuarine bacteria. Prevalence found was low (3.6%) but provided the first hint that integrons could be more prevalent outside clinical settings than previously thought, since less than 1% of the bacteria in environmental samples are believed to be able to grow under lab conditions (Amann *et al.*, 1995). Several other studies concerning integron detection and characterization in bacterial isolates followed this one and contributed to gather knowledge on integron ecology and diversity in several environments. Those included the occurrence of integrons in bacteria isolated from livestock, companion and exotic animals (Goldstein *et al.*, 2001), fish farm environments (Schmidt *et al.*, 2001), irrigation water and sediments (Roe *et al.*, 2003), quaternary ammonium compound-polluted environments (Gaze *et al.*, 2005), uranium-rich environments (Ghauri *et al.*, 2003), animal faecal samples

(Barlow *et al.*, 2004; Barlow *et al.*, 2006; Barlow *et al.*, 2009) and in estuarine environments (Henriques *et al.*, 2006a; Rosewarne *et al.*, 2010). Results obtained suggested that not only integrons are present in all the environments, particularly class 1 integrons, but also highlight for the possibility that heavy metal and quaternary ammonium pollution may co-select for antibiotic-resistance and integron-carrying bacteria.

Investigations have also focused on the prevalence of integrons in bacterial communities using culture-independent approaches, thus overcoming the biases imposed by cultivation-based studies. Nield and co-workers (2001) investigated the presence of integrons in total community DNA samples from environments with no known history of antibiotic exposure. Using primers designed for conserved regions of *intI* genes and *attC* recombination sites, authors detected previously uncharacterized integrons, including three new classes of integrases (Nield *et al.*, 2001). Further investigation on heavy metal contamined sites found six novel *intI* lineages (Nemergut *et al.*, 2004), suggesting that the *intI* gene pool inaccessible by cultivation could be much more diverse than previously thought. Microbial communities inhabiting deep-sea hydrothermal vents, an environment that is devoid of anthropogenic disturbance, have also shown a remarkable diversity of *intI* genes, where authors identified 11 previously undescribed integron classes (Elsaied *et al.*, 2007). These finding suggest that the integrons may be ubiquitous in nature.

More recently, real-time quantitative PCR protocols have been established to estimate integron abundance in DNA samples (Hardwick *et al.*, 2008; Rosewarne *et al.*, 2010; Barraud *et al.*, 2010). Results obtained have shown positive correlations between abundance of *intI1* and ecological conditions (Hardwick *et al.*, 2008) as well as with the presence of heavy metals (Rosewarne *et al.*, 2010).

The strategy designed by Stokes and co-workers (2001) based on the amplification of gene cassettes using degenerated primers targeting *attC* recombination sites in polymerase chain reactions (PCR), allowed the recovery of

entire gene cassettes from environmental DNA from several sources, including seawater, soil and sediment. Similar strategies have been employed by other authors in industrial polluted estuaries (Wright *et al.*, 2008; Koenig *et al.*, 2009), marine environments (Koenig *et al.*, 2008), as well as in deep-sea hydrothermal vents (Elsaied *et al.*, 2007) and wastewaters (**Chapter 2**). In these studies, contrarily to what is observed in clinical settings, few gene cassettes retrieved seem to be involved in antibiotic resistance. This supports the hypothesis that integrons may not be necessarily involved in antibiotic resistance. The majority of sequences retrieved encode novel putative proteins, suggesting that the environmental gene cassette reservoir is vast and diverse, as gene cassette accumulation analyses show no signs of saturation. Attempts to characterize a few ORFs have been made (Nield *et al.*, 2004; Deshpande *et al.*, 2011), confirming that sequences retrieved correspond to functional proteins. The functional characterization of this unknown gene pool may provide interesting insights into bacterial adaptive traits and interaction pathways and may constitute a source of novel enzymes and proteins.

1.4 Scope of this thesis

Along with overconsumption and resulting water shortages, the introduction of substances hazardous to human health into terrestrial water bodies is the most serious threat to water supplies. In addition, as mentioned before, human and animal wastes may contain antibiotics or active intermediates that may persist in effluents. This can potentially increase antibiotic resistance selection as well as the introduction of pathogens and MGEs through wastewater discharges into natural environments with negative impacts on water quality.

Although the presence of antibiotic-degrading bacteria in wastewater treatment plants (WWTPs) favour the breakdown of antibiotic compounds, it may pose a threat to human health if not completely removed during wastewater treatment. The primary goal of wastewater treatment is not to fully eradicate harmful contaminants, but to reduce the organic content of effluents.

Wastewater treatment usually comprises the following steps: primary, secondary, and tertiary treatment (Bitton, 2005). Primary treatment consists of the physical removal of large debris from influent wastewater flow (incoming raw waters). Sequentially, secondary treatment consists in the use of biological processes to remove suspended solids and microorganisms from wastewater. The most common type of secondary treatment is the activated sludge process, which introduces nitrifying microorganisms and high loads of oxygen into wastewater to break down suspended organic materials under aerobic conditions. After the activated sludge process, the wastewater goes into secondary clarifiers to induce the die-off microbial populations and the settling of microbial aggregates, allowing the physical removal of organic material. The secondary treatment processes can remove up to 90% of organic content from wastewaters. Tertiary treatment consists in any additional treatment applied after secondary treatment, such as chlorination, ultraviolet light or ozone disinfection, for elimination of pathogens and control of nutrients (Bitton, 2005). However, tertiary treatment is

seldom applied in wastewater treatment in Portugal, due to its high cost and maintenance requirements.

At the start of this work, the fate of integron–carrying bacteria entering engineered systems and the role of wastewaters in the spreading of antibiotic-resistant bacteria had not been previously investigated. Moreover, in Portugal, the prevalence of integrons outside clinical settings was still poorly understood.

In this context, the main goal of the present work was to evaluate the prevalence, molecular diversity and plasmid-mediated transferability of integrons in bacterial communities inhabiting wastewaters with distinct origins (animal *versus* human).

For that purpose, the following specific aims were established:

(i) to assess the prevalence and diversity of integrons in different wastewaters using culture-independent methodologies (**Chapter 2**);

(ii) to assess the prevalence and diversity of integrons in bacteria as evaluated by culture-dependent methodologies (**Chapters 3**-**4**);

(iii) to evaluate the diversity of promoter sequences regulating gene cassette expression in order to infer on integrase activity and gene cassette dynamics in bacteria from wastewater environments (**Chapter 5**);

(iv) to analyze the association of integron with broad-host-range plasmids that can act as vectors of integron dispersion (**Chapter 6**);

(v) to develop an online database to congregate integron and gene cassette data (**Chapter 7**).

CHAPTER 2

Research in Microbiology (2010) 161:58-66

Insights into the Mobile Gene Pool from Wastewater Environments Using Culture-Independent Approaches

2.1 Introduction

Wastewater effluents are rich environments in what concerns bacterial diversity (Rani *et al.,* 2008; Moura *et al.,* 2009). However, despite the high concentration of organic loads that favour bacterial growth, those complex microbial communities are subjected to strong anthropogenic pressures, such as high levels of pollutant compounds, including detergents (Barnes *et al.,* 2008), heavy metals (Rule *et al.,* 2006), pesticides (Ansari & Malik, 2009) and antimicrobial agents (Brown *et al.,* 2006; Lindgberg *et al.,* 2007).

Bacterial strategies to escape stress conditions include several molecular adaptations, such as the acquisition of exogenous DNA through horizontal gene transfer, enabling bacteria to obtain genes encoding novel or more versatile pathways and promoting bacterial evolution (Davids & Zhang, 2008; Frost *et al.,* 2005).

Genome sequencing accomplishments have brought into light the dissemination of mobile genetic elements (MGEs) on bacterial genomes (Boucher *et al.,* 2007). Although there is some controversy in the classification of integrons as MGEs, since these elements cannot catalyze their transposition, their mobilization can occur mediated by insertion sequences, transposons, integron mobilization units or conjugative plasmids (Mazel, 2006; Poirel *et al.,* 2009). Consequently, integrons definitively contribute to the mobile gene pool. Integrons are present in 9% of the sequenced genomes (Boucher *et al.,* 2007), including Gram-negative and Gram-positive bacteria (**Chapter 7;** Nandi *et al.,* 2004). In addition, complete sequence of environmental plasmids made also clear that BHR-plasmids, which can be transferred and stably maintained in taxonomically distant organisms

(Götz *et al.*, 1996), constitute main vehicles of integron dispersion (Schlüter *et al.*, 2007b). The presence of integrons have been identified in BHR-plasmids belonging to IncP (e.g., R751; Smith & Thomas, 1985), IncN (R46; Stokes & Hall, 1992), IncW (e.g. R388; Swift *et al.*, 1981) and IncQ-like (e.g. pCHE-A; Poirel *et al.*, 2009) incompatibility groups. Since integrons act as platforms of gene cassette capture and expression, these structures together with BHR-plasmids play an important role on the acquisition and spreading of adaptive capabilities to environmental shifts. For instance, the spread of antibiotic resistance, which is commonly mediated by integrons and BHR-plasmids, is seen as a consequence of the misuse of antibiotics in clinical and farming settings (Schlüter *et al.*, 2007b).

The presence of BHR-plasmids carrying antibiotic resistance determinants, as well as genes encoding degradative capabilities and other metabolic functions, has been frequently reported in wastewater environments (Schlüter *et al.*, 2007b; Bahl *et al.*, 2009a). The role of wastewater discharges in the dissemination of integrons encoding undesirable genetic traits into the environment has also been evaluated in a number of recent studies. These studies were focused on bacterial isolates and the majority of gene cassettes observed consisted mostly of antibiotic-resistance encoding genes (**Chapters 2-3**; Zhang *et al.*, 2009). However, the use of cultivation-dependent approaches underestimates the total reservoir of environmental integrons and associated genetic traits present in wastewaters, given that only a small fraction (~1%) of microorganisms can be cultivated in laboratory by standard methods (Amann *et al.*, 1995).

In this work, our aim was to investigate the assortment of promiscuous plasmids, integrons and gene cassettes in wastewater bacterial communities. Two distinct effluents were studied to evaluate changes in the abundance and diversity of those elements depending on the source of wastewaters.

2.2 Material and methods

2.2.1 Sampling and extraction of total community DNA

Sampling was performed in two activated sludge wastewater treatment plants (WWTPs) located in the North of Portugal: one receiving urban wastewaters and the other receiving wastewaters exclusively from a slaughterhouse. Both WWTPs carried out primary and secondary treatments based on activated sludge processes. None carried out disinfection or tertiary treatment. Biochemical oxygen demand (BOD_5), chemical oxygen demand (COD), total suspend solids (TSS) and pH were determined by standard methods (Clescerl *et al.*, 1998).

For total community DNA extraction, water samples were obtained from several points in each raw wastewater. Water samples (25 mL) were filtered through 0.2 μm nylon membranes and membranes were washed with TE buffer to collect cells. DNA was extracted in triplicate and purified using Genomic DNA Purification Kit (MBI Fermentas, Vilnius, Lithuania) with some additional lysing steps (Henriques *et al.*, 2004). Aliquots were loaded onto 0.8% agarose gels and separated by electrophoresis at 80 V for 80 min.

2.2.2 Estimation of total number of bacterial 16S rRNA genes by quantitative PCR (qPCR)

The total number of 16S rRNA genes was estimated using a TaqMan-qPCR assay. The reaction mixture (50 μL) was composed of 1.25 U TrueStart *Taq* and 1x buffer (MBI Fermentas, Vilnius, Lithuania), 2 mM of each deoxynucleoside triphosphate, 2.5 mM $MgCl_2$ and 0.3 mM of each primers and probe (**Table 2.1**). Quantification was carried out in an ABI7000 thermocycler (Applied Biosystems, Warrington, UK) with the following amplification program: one cycle of 95°C for 5 min, and then 40 cycles of 95°C for 15 s, 50°C for 15 s and 60°C for 90 s.

Table 2.1 Oligonucleotides used in this study.

Target	Primer or Probe	Sequence (5'-3')	Positive Control	Annealing Temperature (ºC)	Amplicon size (bp)	Reference
16S rRNA gene (qPCR)	Bac349F Bac806R	AGG CAG CAG TDR GGA AT GGA CTA CYV GGG TAT CTA AT	-	50	460	Takai & Horikoshi, 2000
16S rRNA gene (probe qPCR)	Bac516F	TGC CAG CAG CCG CGG TAA TAC RDA G	-	50	-	Takai & Horikoshi, 2000
16S rRNA gene (PCR-DGGE)	F968GC R1378	GC clamp[a]-AAC GCG AAG AAC CTT AC CGG TGT GTA CAA GGC CCG GGA ACG	-	53	430	Heuer *et al.,* 1997
intI1	intI1F intI1R	CCT CCC GCA CGA TGA TC TCC ACG CAT CGT CAG GC	*Salmonella enterica* (AM237806)	55	280	Kraft *et al.,* 1986
intI2	intI2F intI2R	TTA TTG CTG GGA TTA GGC ACG GCT ACC CTC TGT TAT C	*Escherichia coli* (AF318070)	52	233	Golstein *et al.,* 2001
intI3	intI3F intI3R	AGT GGG TGG CGA ATG AGT G TGT TCT TGT ATC GGC AGG TG	*Klebsiella pneumoniae* (AY219651)	50	600	Golstein *et al.,* 2001
attC elements	HS286 HS287	GGG ATC CTC SGC TKG ARC GAM TTG TTA GVC GGG ATC CGC SGC TKA NCT CVR RCG TTA GSC	*Salmonella enterica* (AM237806)	55	variable	Stokes *et al.,* 2001
IncN plasmids	rep1 rep2	AGT TCA CCA CCT ACT CGC TCC G CAA GTT CTT CTG TTG GGA TTC CG	pRN3	55	165	Götz *et al.,* 1996
IncP-1 plasmids	trfA_fw trfA_rev	TTC ACS TTC TAC GAG MTK TGC CAG GAC GWC AGC TTG CGG TAC TTC TCC CA	pRP4 (α); R751(β); pKJ5(ε)	60	281	Bahl *et al.,* 2009a
IncQ plasmids	oriV 1 oriV 2	CTC CCG TAC TAA CTG TCA CG ATC GAC CGA GAC AGG CCC TGC	pRSF1010	53	436	Götz *et al.,* 1996
IncW plasmids	oriV 1 oriV 2	GAC CCG GAA AAC CAA AAA TA GTG AGG GTG AGG GTG CTA TC	pR388	57	1140	Götz *et al.,* 1996
pCR®2.1	M13Rev T7 promoter	CAG GAA ACA GCT ATG ACC TAA TAC GAC TCA CTA TAG GG	pCR®2.1	51	1467	Invitrogen, Carlsbad, CA, USA

[a]GC clamp: CGCCCGGGGCGCGCCCCGGGCGGGGCGGGGGCACGGGGGG

Standard curves were constructed using serial dilutions of purified PCR products of 16S rRNA genes from *Escherichia coli* (Heuer & Smalla, 2007).

2.2.3 DGGE analysis of PCR-amplified 16S rRNA *gene fragments*

Differences on bacterial community structure from both wastewaters were evaluated by 16S rDNA-DGGE analysis. Amplification of bacterial 16S rRNA gene fragments (**Table 2.1**) was carried out as described previously (Heuer *et al.*, 1997). Amplicons were separated by DGGE in a 26–58% denaturing gradient (100% denaturant is defined as 7M urea and 40% (v/v) formamide) with an additional acrylamide gradient from 6 to 9% to improve band resolution (Cremonesi *et al.*, 1997). Electrophoresis was performed in a DCode™ Universal Mutation Detection System (BioRad, Hercules, CA, USA) in 1x TAE buffer (20 mM Tris, 10 mM acetic acid, 0.5 mM EDTA, pH 8.0), at 58 ^{o}C, for 6 h and at a constant voltage of 220 V. Gel was silver stained, dried and documented. Gel image analysis was performed with GelCompar II version 4.6 (Applied Maths, Sint-Martens Latem, Belgium). Calculation of similarities of DGGE band profiles was based on Pearson coefficient and a dendrogram was obtained by unweighted pair group mean average (UPGMA) cluster analysis.

DGGE band profiles were also examined using Shannon-Weaver index of diversity (Shannon & Weaver, 1949) and the equitability index (Pielou, 1975).

2.2.4 Detection of intI genes and broad-host-range plasmid specific sequences by PCR and Southern hybridization

PCR amplification of *intI1*, *intI2* and *intI3* genes coding for integrases of class 1 to 3, respectively was performed separately by using primers listed in **Table 2.1,** as described in detail in **Chapter 3**.

Amplification of BHR-plasmid specific sequences belonging to IncP-1 (*trfA*), IncN (*rep*), IncW (*oriV*) and IncQ (*oriV*) incompatibility groups (**Table 2.1**) was performed as described previously (Götz *et al.*, 1996), using 2.5 U *Taq* polymerase per reaction instead of 5 U.

Aliquots of 10 μL of PCR products were run in agarose gels and DNA was transferred overnight to nylon membranes (Hybond-N; Amersham, Freiburg, Germany) according to standard protocols (Sambrook & Russel, 1989). Hybridizations with digoxigenin-labelled probes were performed in middle stringency conditions (homology 75-100%) in 20% formamide hybridization buffer at 62 ^{o}C, following manufacturer's recommendations (Roche Diagnostic, Mannheim, Germany). Positive and negative controls were included in all the experiments to confirm the specificity of detection.

2.2.5 Construction of gene cassette libraries and sequence analysis

Gene cassettes were recovered from total community DNA by PCR amplification using primers HS286/HS287 (**Table 2.1**) targeting the *attC* recombination sites that flank gene cassettes. Amplified DNA fragments were cloned into pCR®2.1 vector (Invitrogen, Carlsbad, CA, USA) and the constructs were transformed into *E. coli* TOP10F' competent cells (Invitrogen, Carlsbad, CA, USA). Positive clones were selected randomly and amplified with primers M13R/T7 (**Table 2.1**). Inserts were digested with *HaeIII* (MBI Fermentas, Vilnius, Lithuania) and clones displaying different restriction profiles were subsequently sequenced by StabVida (Oeiras, Portugal).

Sequence analyses of cloned PCR products were performed using ORF Finder (http://www.ncbi.nlm.nih.gov/projects/gorf/orfig.cgi) and BLASTP (http://www.ncbi.nlm.nih.gov/blast.cgi) software to search for putative open reading frames and protein matches in GenBank (http://www.ncbi.nlm.nih.gov). Operational taxonomic units (OTUs) were defined as gene cassettes that shared

100% amino acid sequence similarity. Species diversity within libraries and accumulation curves were obtained using EstimateS version 7.5 (http://purl.oclc.org/estimates). The percentage of coverage (C) was calculated using the equation C=1-(n/N)*100%, where n is the number of OTUs containing only one sequence and N is the number of clones examined (Kemp & Aller, 2004).

2.2.6 Accession numbers

Nucleotide sequences of gene cassette regions were deposited in GenBank under the accession numbers FJ541310-FJ541363 (clones MM.RW) and FJ541364-FJ541429 (clones ER.RW).

2.3 Results

2.3.1 Sample characterization

Chemical parameters determined for the raw waters sampled in the slaughterhouse (MM.RW) wastewater treatment plant and in the urban (ER.RW) wastewater treatment plant are shown in **Table 2.2**. Comparing to urban effluent, slaughterhouse raw waters showed higher COD, BOD_5 and TSS values and lower pH.

Table 2.2 Characterization of urban (ER.RW) and slaughterhouse (MM.RW) wastewater effluents in terms of pH, chemical oxygen demand (COD), biological oxygen demand (BOD_5) and total suspended soils (TSS).

Sampling Site	pH (at 20ºC)	COD ($g.L^{-1}$ O_2)	BOD_5 ($g.L^{-1}$ O_2)	TSS ($g.L^{-1}$)
MM.RW	6.7	4.1	2.6	2.3
ER.RW	7.28	2.9	0.38	0.32

2.3.2 16S rRNA gene quantification and PCR-DGGE analysis of bacterial community

The number of 16S rRNA genes was estimated by qPCR to normalize the amount of total DNA used for hybridization experiments. PCR quantification results indicated that the amounts of 16S rRNA gene copies detected were in the same order of magnitude (10^9) among samples and replicates (**Table 2.3**).

Cluster analysis of DGGE profiles obtained from wastewater samples indicated clear differences in bacterial diversity and structure associated with the type of effluent. Bacterial community structure in urban (ER.RW) and slaughterhouse (MM.RW) wastewaters shared only 23% of similarity. Profiles were stable among replicates (**Figure 2.1**).

Shannon (H) and equitability (E) numerical indexes demonstrated that bacterial communities in urban waters ER.RW (H= 5.12 ± 0.04; E= 0.93 ± 0.01) were more diverse and with even distribution of taxa while MM.RW samples showed lower bacterial diversity and pronounced dominance of taxa (H= 2.75 ± 0.49; E= 0.59 ± 0.11).

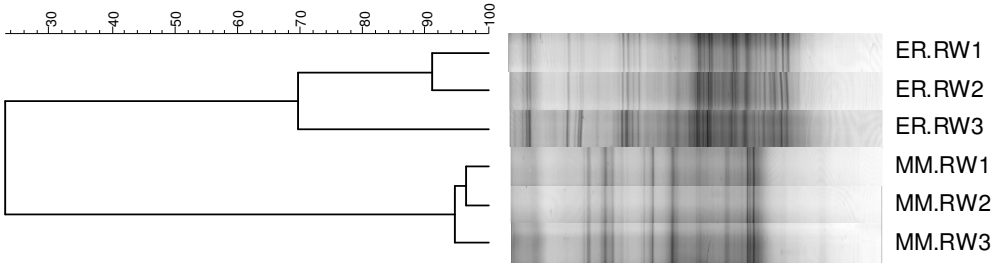

Figure 2.1 UPGMA dendrogram generated from bacterial DGGE profiles based on Pearson similarity correlation. Samples are from the raw waters from urban (ER.RW) and (MM.RW) wastewaters treatment plants. Numbers 1-3 correspond to replicates.

2.3.3 Detection of intI genes and BHR-plasmid sequences in total community DNA

Detection of *intI* genes and BHR-plasmids by PCR amplification and hybridization in total community DNA revealed that urban and slaughterhouse wastewaters shared a common pool of mobile elements (**Table 2.3**). Integrase encoding genes belonging to classes 1, 2 and 3 were detected in both effluents. Additionally, all plasmid-specific sequences assessed (IncP-1, IncN, IncW and IncQ incompatibility groups) were present on both effluents. However, among IncP-1 subgroups, differences were observed depending on the type of effluent: IncP-1ε and IncP-1β subgroups were prevalent in urban wastewaters while IncP-1α was prevalent in slaughterhouse wastewaters (**Table 2.3**).

2.3.4 Analysis of gene cassette clone libraries

A total of 186 clones were randomly picked from gene cassette libraries derived from total community DNA of MM.RW and ER.RW raw waters. Clones contained inserts ranging from 0.2 - 1.0 kb in MM.RW and 0.3 - 0.9 kb in ER.RW. Clones containing inserts smaller than 300 bp were excluded from further characterization. Restriction fragment length polymorphism analyses resulted in 67 distinct profiles in MM.RW (51 of those with only one representative) and 74 profiles in ER.RW (59 of those with only one representative), which were further sequenced. Most of the cloned inserts contained single open reading frames (ORFs).

Sixty-four and 53 ORFS were identified in MM.RW and ER.RW libraries, respectively. No ORFs were found to be common to both libraries. The translation of the nucleotide sequences of those ORFs reveals that they may encode several putative proteins (**Table 2.4-2.5**), although 32% of which possessed no identified homologous in GenBank database. This was true for both effluents. Protein

sequences deduced from MM.RW ORFs ranged from 49 to 162 amino acids, representing putative enzymes involved in aminosugars metabolism, cell wall synthesis, transcriptional regulation, toxin biogenesis and virulence, biosynthesis of fatty acids, cellular energy conversion and antifolate resistance (**Table 2.4**). Protein sequences deduced from ER.RW ORFs ranged from 54 to 140 aa and shared similarity with proteins involved in motility, gene regulation, membrane structure, intercellular signalling and secretion pathways, synthesis of cellulose, antibiotic biosynthesis and resistance (**Table 2.5**).

Library coverage (37.23% for ER.RW and 38.55% for MM.RW clone libraries) as well as the absence of asymptotic rarefaction curves (**Figure 2.2**) suggest the presence of an extensive and diverse gene cassette pool in wastewater samples. In addition, estimations using Chao1 richness index indicate that likely more than 200 different gene cassettes (100% sequence similarity) might be present in urban and slaughterhouse clone libraries. Therefore, more sequencing would be needed for both libraries to capture the complete diversity of gene cassettes.

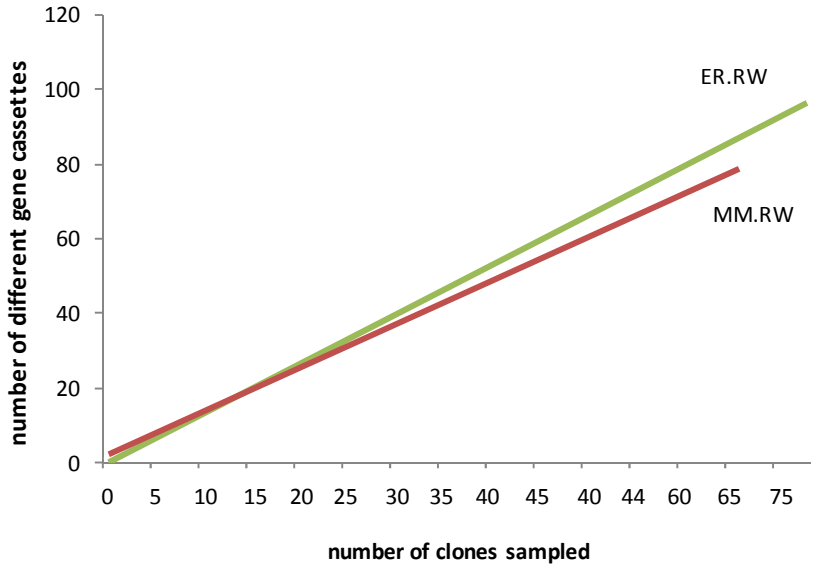

Figure 2.2 Rarefaction analysis comparing gene cassette diversity in ER.RW (urban wastewater) and MM.RW (slaughterhouse wastewater) clone libraries.

Table 2.3 Detection of mobile genetic elements in urban (ER.RW) and slaughterhouse (MM.RW) wastewaters by Southern hybridization: + hybridization; +++ very strong hybridization.

Sample	IncP-1α *trfA*	IncP-1β *trfA*	IncP-1ε *trfA*	IncN *rep*	IncQ *oriV*	IncW *oriV*	*intI1*	*intI2*	*intI3*	16S rDNA gene copy number.L^{-1} wastewater
ER.RW 1	+	+++	+++	+	+++	+	+++	+++	+++	2.77×10^9
2	+	+++	+++	+	+++	+	+++	+++	+++	1.44×10^9
3	+	+++	+++	+	+++	+	+++	+++	+++	2.48×10^9
MM.RW 1	+++	+	+	+++	+++	+++	+++	+++	+++	4.25×10^9
2	+++	+	+	+++	+++	+++	+++	+++	+++	3.38×10^9
3	+++	+	+	+++	+++	+++	+++	+++	+++	4.03×10^9

Table 2.4 Gene cassette products with database matches retrieved from MM.RW clone library (slaughterhouse wastewaters).

ORF	Size (aa)	Closest match (e-value)	Organism (accession number)
MM.RW01	94	Hypothetical protein VSWAT3_12332 (0.36)	*Vibrionales* bacterium SWAT-3 (EDK30857)
MM.RW02	102	Putative integron gene cassette protein (8E-55)	Uncultured bacterium (CAP48370)
MM.RW03	104	Putative integron gene cassette protein (3E-22)	Uncultured bacterium (CAP49045)
MM.RW04	153	Hypothetical protein (0.008)	Uncultured bacterium (BAF45167)
MM.RW05	88	Hypothetical protein (5.8)	*Streptomyces kanamyceticus* (BAE95516)
MM.RW06	116	Hypothetical protein Sfri_1661 (7.0E-12)	*Shewanella frigidimarina* (ABI71512)
MM.RW07	162	Hypothetical protein vspid_17095 (2.0E-57)	*Verrucomicrobium spinosum* (ZP_02928387)
MM.RW08	148	NADH dehydrogenase subunit 5 (1.7)	*Sphingonotus caerulans* (ABY89046)
MM.RW09a	88	Pteridine reductase (1.0E-24)	*Psychrobacter arcticus* (YP_264651)
MM.RW09b	49	Phosphoglucosamine mutase (5.6)	*Acidobacteria* bacterium (ABF39281)
MM.RW14a	74	Hypothetical protein P0648C09.8 (7.7)	*Oryza sativa Japonica* (BAB86219)
MM.RW14b	140	Hypothetical protein Mpe_A0693 (1.0E-33)	*Methylibium petroleiphilum* (YP_001019890)
MM.RW18	140	Hypothetical protein Mpe_A0693 (1.0E-33)	*Methylibium petroleiphilum* (YP_001019890)
MM.RW19	83	Hypothetical protein (6.2)	*Monosiga brevicollis* (EDQ86645)
MM.RW20a	73	Hypothetical protein PROVALCAL_00955 (9.0)	*Providencia alcalifaciens* (EEB46945)
MM.RW20b	61	Acetyltransferase (8.0E-11)	*Aeromonas salmonicida* (YP_001142065)
MM.RW20c	62	Fis family transcriptional regulator (9.3)	*Syntrophobacter fumaroxidans* (ABK19434)
MM.RW21	137	Hypothetical protein CY0110_07274 (0.084)	*Cyanothece* sp. (EAZ89978)
MM.RW22a	140	Hypothetical protein Mpe_A0693 (1.0E-33)	*Methylibium petroleiphilum* (ABM93655)
MM.RW22b	74	Hypothetical protein P0648C09.8 (7.7)	*Oryza sativa Japonica* (BAB86219)
MM.RW23a	87	Acetyltransferase (2.0E-18)	*Aeromonas salmonicida* subsp. *Salmonicida* A449 (ABO90317)
MM.RW23b	62	Fis family transcriptional regulator (9.3)	*Syntrophobacter fumaroxidans* (ABK19434)
MM.RW24	49	Phosphoglucosamine mutase (5.6)	*Acidobacteria* bacterium (ABF39281)
MM.RW25	124	Hypothetical protein eba93 (0.067)	*Azoarcus* sp. (CAI06173)
MM.RW28a	82	Hypothetical protein CHGG_05848 (2.8)	*Chaetomium globosum* (EAQ89229)
MM.RW28b	101	Hypothetical protein CPS_1444 (6.0)	*Colwellia psychrerythraea* (AAZ26761)
MM.RW29	132	Hypothetical protein dalkdraft_4890 (4.0E-17)	*Desulfatibacillum alkenivorans* (EDQ29106)
MM.RW37	116	Hypothetical protein Sfri_1661 (7.0E-12)	*Shewanella frigidimarina* (ABI71512)
MM.RW45	115	RTX toxin rtxa (0.53)	*Vibrio cholerae* (EAZ74142)
MM.RW46	148	Hypothetical protein HCH_10017 (1.0E-19)	*Hahella chejuensis* (ABC31270)
MM.RW47	104	Hypothetical protein XCC0751 (4.0E-06)	*Xanthomonas campestris* (AAM40066)
MM.RW48	91	Hypothetical protein VIBHAR_01711 (1.3)	*Vibrio harveyi* (ABU70680)
MM.RW48	97	Hypothetical protein (2.0E-29)	*Pseudomonas aeruginosa* (CAG23928)
MM.RW49	112	Hypothetical protein VC274080_A0340 (4.0E-14)	*Vibrio cholerae* (EAX59829)
MM.RW51a	144	Gp38 (2.0E-18)	Enterobacteria phage psp3 (AAN08402)
MM.RW51b		Putative peptidoglycan binding domain (2.4)	*Brucella abortus* (ACD72866)
MM.RW54a	140	Hypothetical protein Mpe_A0693 (1.0E-33)	*Methylibium petroleiphilum* (ABM93655)
MM.RW54b	74	Hypothetical protein P0648C09.8 (7.7)	*Oryza sativa Japonica* (BAB86219)
MM.RW57	52	Conserved hypothetical protein (10)	*Arthrobacter chlorophenolicus* (EDS60957)
MM.RW59	124	Hypothetical protein eba93 (0.068)	*Azoarcus* sp. (CAI06173)
MM.RW60	94	Hypothetical protein VSWAT3_1233 (0.33)	*Vibrionales* bacterium SWAT-3 (EDK30857)
MM.RW61	148	Hypothetical protein HCH_10017 (1.0E-19)	*Hahella chejuensis* (ABC31270)
MM.RW64	112	Hypothetical protein VC274080_A0340 (4.0E-14)	*Vibrio cholerae* (EAX59829)
MM.RW66	137	Putative wall-associated kinase (8.5)	*Oryza sativa Japonica* (EAZ23865)
MM.RW67	97	Hypothetical protein Rmet_6155 (2.0E-12)	*Ralstonia metallidurans* (ABF13014)
MM.RW68	124	Hypothetical protein eba93 (0.068)	*Azoarcus* sp. (CAI06173)
MM.RW69	137	Hypothetical protein CY0110_07274 (0.084)	*Cyanothece* sp. CCY0110 (EAZ89978)
MM.RW70a	120	Hypothetical protein CBG18709 (1.5)	*Caenorhabditis briggsae* (CAP36112)
MM.RW70b	74	Hypothetical protein (7.4)	*Entamoeba histolytica* (EAL49060)
MM.RW81	89	Putative integron gene cassette protein (4.0E-39)	Uncultured bacterium (CAP48271)

Table 2.5 Gene cassette products with database matches retrieved from ER.RW clone library (urban wastewaters).

ORF	Size (aa)	Closest match (e-value)	Organism (accession number)
ER.RW1	74	Cellulose synthase-1-like protein (0.65)	*Oryza sativa Japonica* (BAC99567)
ER.RW2	133	Antibiotic biosynthesis monooxygenase (4.0E-37)	*Chloroflexus aurantiacus* (ABY35652)
ER.RW3	74	Cellulose synthase-1-like protein (0.65)	*Oryza sativa Japonica* (BAC99567)
ER.RW4	89	Putative integron gene cassette protein (2.0E-45)	Uncultured bacterium (CAP48717)
ER.RW6	77	Pilt protein-like (3.0E-20)	*Nitrococcus mobilis* Nb-231 (ZP_01128839)
ER.RW8	133	Putative integron gene cassette protein (2.0E-64)	Uncultured bacterium (CAP48875)
ER.RW10	128	Orf152egc145 (1.0E-18)	Uncultured bacterium (AF421332)
ER.RW11	91	Putative integron gene cassette protein (7.0E-24)	Uncultured bacterium (CAP48097)
ER.RW12a	73	Hypothetical protein aael_AAEL003131 (1.0)	*Aedes aegypti* (XP_001656329)
ER.RW12b	69	Conserved hypothetical protein (2.4)	*Pyrenophora tritici-repentis* (EDU40194)
ER.RW13	106	4-amino-4-deoxychorismate lyase (6.3)	*Propionibacterium acnes* (AAT83963)
ER.RW14a	73	Similar to flagella synthesis protein flgn (7.0)	Candidatus *Kuenenia stuttgartiensis* (CAJ74304)
ER.RW14b	54	TPA: zinc finger protein (2.6)	*Ciona intestinalis* (FAA00104)
ER.RW15a	90	Hypothetical protein MAE_2561 (7.0E-16)	*Microcystis aeruginosa* NIES-843 (BAG02383)
ER.RW15b	94	Conserved hypothetical protein (7.0E-16)	*Thauera* sp. MZ1T (EDS56560)
ER.RW18	75	General secretion pathway protein G (1.7)	*Methylocella silvestris* (EDT52606)
ER.RW19	75	General secretion pathway protein G (1.7)	*Methylocella silvestris* (EDT52606)
ER.RW20	112	Hypothetical protein BACSTE_01949 (0.32)	*Bacteroides stercoris* (EDS15447)
ER.RW21	60	Putative integron gene cassette protein (2.0E-23)	Uncultured bacterium (CAP48777)
ER.RW22	77	Putative integron gene cassette protein (4.0E-17)	Uncultured bacterium (CAP48790)
ER.RW30	89	Putative integron gene cassette protein (5.0E-46)	Uncultured bacterium (CAP48717)
ER.RW34	140	Ompa domain-containing protein (1.8)	*Verminephrobacter eiseniae* (ABM56789)
ER.RW37	70	Hypothetical protein NCU05966 (9.5)	*Neurospora crassa* (EAA29344)
ER.RW40	77	Hypothetical protein BACDOR_02507 (4.0)	*Bacteroides dorei* (EEB25041)
ER.RW42	106	Hypothetical protein RSP_0419 (1.2)	*Rhodobacter sphaeroides* (ABA79595)
ER.RW43	74	Conserved hypothetical protein (4.5)	*Pediculus humanus corporis* (EEB18218)
ER.RW44	97	Hypothetical protein (3.0E-20)	*Pseudomonas stutzeri* (ABV54364)
ER.RW46	140	Ompa domain-containing protein (0.90)	*Verminephrobacter eiseniae* (ABM56789)
ER.RW52	89	Putative integron gene cassette protein (2.0E-45)	Uncultured bacterium (CAP48717)
ER.RW53	60	Putative integron gene cassette protein (9.0E-23)	Uncultured bacterium (CAP48777)
ER.RW55	77	Hypothetical protein BACDOR_02507 (3.6)	*Bacteroides dorei* DSM 17855 (EEB25041)
ER.RW56	82	Hypothetical protein NE1583 (2.0E-28)	*Nitrosomonas europaea* ATCC 19718 (CAD85494)
ER.RW58	161	Putative integron gene cassette protein (5.0E-69)	Uncultured bacterium (CAP48335)
ER.RW59	143	Integrase (0.9)	*Klebsiella pneumoniae*
ER.RW60	84	Putative protein-S-isoprenylcysteine methyltransferase-like (4.0E-20)	*Polaromonas* sp. JS666 (ABE45276)
ER.RW67	66	Hypothetical protein KAOT1_15678 (0.006)	*Kordia algicida* (ZP_02161471)
ER.RW70	77	Hypothetical protein BACDOR_02507 (3.6)	*Bacteroides dorei* DSM 17855 (EEB25041)
ER.RW71	57	Putative transmembrane protein (0.22)	*Stenotrophomonas maltophilia* (CAQ45356)
ER.RW76	95	Hypothetical protein OM2255_20457 (5.0E-10)	Alpha proteobacterium HTCC2255 (ZP_01448177)
ER.RW85	127	Camphor resistance protein crcb (2.0E-26)	*Methylobacillus flagellatus* (ABE49489)
ER.RW89a	123	Hypothetical protein P700755_16504 (8.0E-15)	*Psychroflexus torquis* (ZP_01255011)
ER.RW89b	58	Nucleoprotein (1.8)	*Influenza A virus* (AAA43241)
ER.RW93	73	Putative integron gene cassette protein (3.0E-19)	Uncultured bacterium (CAS02883)
ER.RW96	105	Hypothetical protein Z2311 (0.15)	*Escherichia coli* O157:H7 (NP_287750)

2.4 Discussion

The aim of this work was to assess the prevalence and diversity of MGEs in two wastewater effluents in Portugal by using cultivation-independent approaches. Two distinct WWTP, one receiving domestic wastewaters and other receiving wastewaters from an animal slaughterhouse, were sampled in order to also gain insights on the abundance and diversity of the mobile gene pool depending on type of effluent. The urban WWTP serves a city with nearly 40.000 inhabitants and collects waters from a very large basin, where bacteria released can reside presumably for longer and more variable periods in nutrient-diluted water suspension. On the other hand, the slaughterhouse WWTP yields wastewaters locally processed containing high loads of blood, organic compounds and particles.

Slaughterhouse wastewaters possessed higher oxygen demands and particles in suspension and lower pH comparing to the urban effluent (Table 2.1). Bacterial communities inhabiting the two WWTPs shared less than 25% of similarity as revealed by DGGE analysis. Mathematic indexes used suggest that slaughterhouse effluent's bacterial community has lower diversity and a dominant structure, contrarily to urban wastewaters that showed more diversity and evener communities. Those statistics together with physicochemical data may indicate that bacterial communities on slaughterhouse effluents are subject to stronger selective pressures.

It is also known that the presence of solids in water as well as high bacterial densities promote biofilm formation and the occurrence of horizontal gene transfer events (Sørensen *et al.*, 2005, Schlüter *et al.*, 2007b). For those reasons, it was expected to observe a wider diversity of integrase genes and plasmid sequences in samples from slaughterhouse wastewaters. Surprisingly, all integrase encoding genes and specific plasmid sequences searched were found in both wastewaters, revealing the presence of an extensive diversity of sequences in both environments.

IncP-1 plasmids are thought to be the most promiscuous plasmids, and are widely spread in several and distinct environments, from soil and manure (Malik *et al.,* 2008; Ansari *et al.*, 2008; Binh *et al.*, 2008; Götz *et al.,* 1996) to aquatic environments, including wastewaters (Inoue *et al.*, 2007; Dröge *et al.*, 2000). IncN, IncQ and IncW plasmids were first described in clinical settings and their presence in environmental samples has been reported in manure and soil (Malik *et al.,* 2008; Ansari *et al.*, 2008; Binh *et al.*, 2008; Götz *et al.,* 1996). Their presence was never detected in riverine waters (Inoue *et al.*, 2007) neither in marine (Sobecky *et al.*, 1997) or wastewater environments (Inoue *et al.*, 2007; Dröge *et al.*, 2000) and therefore, their presence in aquatic systems seemed to be remote. In this study, IncN, IncQ and IncW specific sequences were detected in both wastewaters, despite the fact that bacterial and nutrient loads as well as the source of microbes in both effluents were rather different. Urban waters contain mostly bacteria from the human intestinal biota, while in slaughterhouse effluents most bacteria come from different origins of the animal microbiota, including skin, bones and digestive tract. These results raise a greater concern and draw attention to the lack of studies regarding BHR-plasmid dispersion in Portugal, in both clinical and natural environments.

In this study, it was also possible to detect the presence of sequences representative of integrases belonging to classes 1 to 3 in all samples. Integron integrases seem to be ubiquitous in nature as they are often recovered from clinical and natural environments, including environments subjected to extreme selective pressures such as high-salt (Ghauri *et al.,* 2006) and uranium-rich environments (Ghauri *et al.,* 2003), deep-sea hydrothermal vents (Elsaied *et al.,* 2007), heavy metal-contaminated mine tailings (Nemergut *et al.,* 2004) and polluted estuarine waters (Henriques *et al.,* 2006; Wright *et al.,* 2008). Integrase genes belonging to class 1to 3 have also been detected in bacterial isolates from wastewaters (**Chapter 2-3**; Tennstedt *et al.*, 2003; Xu *et al.*, 2007), although *intI1* genes were prevalent in these environments. Interestingly, in this study no differences were found in the prevalence among integrase genes classes.

The gene cassette reservoir present in urban and slaughterhouse wastewaters was also investigated. No gene cassettes were common to both libraries. These results contradict those from Wright *et al.* (2008) that reported higher abundance of *intI1* genes in contaminant-exposed communities from estuarine and riverine microhabitats, but found no differences in what concerned gene cassette content depending on the level of contamination. Our results suggest that wastewaters are important reservoirs of MGEs, but the specific traits they carry may be selected depending on the type of selective pressures undergone. Genes recovered from clone libraries showed regions of low homology to DNA sequences involved in several functions, such as cellular metabolism, motility and virulence. Thus, results obtained extend the role of gene cassettes further than antibiotic resistance and as sources of protein diversity (Nemergut *et al.*, 2004).

In both libraries homologies to putative integron gene cassette proteins previously reported in contaminated marine sediments (Koenig *et al.*, 2008) were also found, suggesting that these sequences may encode proteins that play an important role in bacterial adaptation to intensive selective pressures characteristic from these environments.

Nevertheless, the majority of cloned gene cassettes seemed to be novel as no homologous in GenBank database were indentified. Our results show that wastewaters environments promote the development of bacterial communities containing a vast and not yet characterized pool of open reading frames captured as gene cassettes and thus susceptible of mobilization and expression. It is important to elucidate the role of these unknown gene cassettes in these particular environmental conditions and whether these conditions promote gene expression and/or integron dissemination.

The simultaneous assess to integron and plasmid specific sequences using PCR-based approaches allowed their detection regardless the host culturability and the presence of selectable markers, contributing to a better understanding of their dispersion in complex bacterial communities such as those inhabiting wastewaters.

Although this study provides only a snapshot of the complex and rich genetic reservoir present in wastewater environments, it shows that wastewater environments promote the development of bacterial communities that support and bring together different types of molecular elements that in association are main players of bacterial adaptation and evolution.

In addition, the presence of putative proteins with potential biotechnological applications justifies further work in gene cassette characterization and stresses the importance of wastewaters gene cassette reservoirs as sources of functionally diverse proteins.

CHAPTER 3

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Prevalence and Diversity of Integrons in Bacteria Isolated from a Slaughterhouse Wastewater Treatment Plant

3.1 Introduction

Wastewater treatment of industrial, agricultural, and domestic discharges plays an essential role in addressing the worldwide problem of increasing water pollution. Wastewater treatment allows waters to be reused for irrigation in agriculture or released directly into the aquatic environments. Sludge produced throughout the detoxification processes may also be used afterwards as fertilizers.

The presence of antibiotic resistant bacteria in effluents (Goñi-Urriza *et al.*, 2000; Schwartz *et al.*, 2003; da Costa *et al.*, 2006) as well as high levels of antibiotic compounds (Lindberg *et al.*, 2007) in wastewater treatment plants (WWTPs) have been addressed in several studies, creating a growing concern about its impact on animal and human health. Slaughterhouses generate large wastewater volumes consisting mostly of blood, fecal matter, oils and greases (Pozo *et al.*, 2003). These conditions may exert selective pressure on sewage bacteria, providing the opportunity for further dissemination of undesirable genetic elements by horizontal gene transfer. However, little is known regarding the presence and dissemination of integrons in WWTPs.

The frequent association of integrons with mobile genetic elements, such as transposons and conjugative plasmids, contributes to their dissemination due to horizontal gene transfer events (Hall *et al.*, 1999; Holmes *et al.*, 2003). Previous studies have been performed in order to access the prevalence of integrons in several natural environments. In estuaries, class 1 integrons were found in 3.6% of Gram-negative bacteria and incidences of 25% and 3.6% of integrons from class 1 and 2, respectively, were reported in ampicillin-resistant *Enterobacteriaceae* and *Aeromonas/Pseudomonas* isolates (Rosser & Young, 1999; Henriques *et al.*, 2006). In irrigation water and sediments, incidences of 4.18% and 0.31%, respectively, for

class 1 and 2 integrons were found among *Escherichia coli* isolates (Roe *et al.*, 2003). Class 1 integrons were also reported in 3.8% of Gram-positive and Gram-negative isolates from quaternary ammonia compounds polluted environments (Gaze *et al.*, 2005). As hypothesised, these environmental reservoirs of integron-carrying bacteria may be linked to run-offs from agriculture fields and/or sewage inputs (Rosser & Young, 1999; Roe *et al.*, 2003; Gaze *et al.*, 2005; Henriques *et al.*, 2006).

In this chapter, the presence and dissemination of integrons in a slaughterhouse wastewater environment were investigated at different stages of the treatment process, in order to gather data pertaining to the contribution of these environments to horizontal gene transfer and spread of antibiotic resistance determinants.

3.2 Material and methods

3.2.1 Sampling and bacterial isolation

Sampling was performed in October 2005 in a WWTP located in Mirandela, Portugal, that receives wastewater exclusively from a slaughterhouse. Treatment consists of an activated sludge process and includes the following steps: mechanical screening, fat separation, equalization (homogenization), biological aeration, pH correction and precipitation. Sludge produced is dried and further used as a fertilizer in agriculture.

Water samples were collected from raw water (RW), homogeny tank (HT), aeration tank (AT), sludge recirculation (SR) and final effluent (FE). Biochemical oxygen demand (BOD_5), chemical oxygen demand (COD), total suspend solids (TSS), oil and grease content and pH were determined by standard methods (Clescerl *et al.*, 1998).

Serial decimal dilutions of water samples were prepared in 0.9% NaCl. Undiluted or diluted samples (10 mL) were filtered through 0.45 μm-pore size

cellulose sterile filters (Pall Life Sciences, MI, USA). Filters were placed onto agar plates selective for *Aeromonas* (Glutamate starch phenol red (GSP) agar, Merck, Darmstadt, Germany) and *Enterobacteriaceae* (MacConkey agar, Merck, Darmstadt, Germany). Duplicate sets of plates were incubated for 24 h at 30ºC and 37°C. All individual colonies used in further experiments were picked from the following dilutions: 10^{-3} and 10^{-2} in RW, 10^{-3} and 10^{-1} in HT, 10^{-3} and 10^{-1} in AT, 10^{-3} and 10^{-2} in SR, 10^{-2} and 10^{-1} in FE, from MacConkey and GSP agar, respectively.

3.2.2 Integron screening by dot-blot hybridization

Integrase genes *intI1*, *intI2* and *intI3* were amplified from genomic DNA of the positive control strains *Salmonella enterica* serovar Typhimurium (*intI1*$^{+}$, AM237806), *E. coli* (*intI2*$^{+}$, accession no. AF318070) and *Klebsiella pneumoniae* (*intI3*$^{+}$, accession no. AY219651), as described previously (Correia *et al.*, 2003; Henriques *et al.*, 2006), using the primer pairs listed in Table 3.1. The amplification products were labelled by incorporation of digoxygenin-11-dUTP (Roche Molecular Biochemicals, Indianapolis, USA) during polymerase chain reaction (PCR). Isolates were screened by dot-blot hybridization. Bacterial colonies were picked from fresh Luria-Bertani agar plates and boiled in 20 µL of sterile distilled water for 15 min at 100°C. Cell suspensions were denatured with NaOH 0.5 M, incubated at 50ºC for 5 min, and equilibrated in 20x SSC. Denatured DNA was transferred onto positively charged nylon membranes (Hybond N+; Amersham, Freiburg, Germany) using Minifold I system (Schleicher and Schuell, Dassel, Germany) and subsequently cross-linked under UV irradiation for 5 min. Hybridizations were performed overnight in 50% formamide hybridization buffer at 42ºC and detections were carried out using the DIG Nucleic Acid Detection Kit following the instructions provided by the manufacturer. Positive and negative controls were included in all experiments to confirm the specificity of detection. Positive detections were further confirmed by PCR to exclude the presence of false-positive results.

3.2.3 Molecular typing and phylogenetic affiliation of intI-positive isolates

Molecular typing of integrase-positive isolates was performed by REP-PCR, using primers REP1R and REP2I (**Table 3.1**) to amplify repetitive extragenic palindromic sequences from 1 µL of cell suspension prepared in 100 µL of distilled water (suspension turbidity equivalent to a McFarland 1.0 standard). The composition of reaction mixtures, the PCR program and gel analysis were as described previously (Henriques *et al.*, 2006).

Isolates displaying different REP-patterns were subsequently identified by 16S rRNA gene sequencing analysis. Amplification was performed with universal bacterial primers 27F and 1492R (**Table 3.1**), as described previously (Lane, 1991). Identification of *Aeromonas* isolates to the species level was achieved by partial amplification (~ 950 bp) and sequencing of the *gyrB* gene (Yáñez *et al.* 2003). PCR products were purified with Jetquick PCR Product Purification Spin Kit (Genomed, Löhne, Germany). Sequencing reactions were carried out using an ABI PRISM® BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems, Foster City, California, USA). Samples were then analysed in an automatic DNA sequencer (ABI PRISM® 310 Genetic Analyser, PE Applied Biosystems). Sequence similarity searches and phylogenetic affiliations were performed using the BLAST program (Altschul *et al.*, 1997).

3.2.4 Integron characterization

Integron characterization was carried out through PCR amplification with primer pairs targeting integron variable regions (Table 3.1), as described previously (White *et al.*, 2001). PCR products with the same size were digested overnight with 1 U of *Hae*III (GG↓CC; MBI Fermentas, Vilnius, Lithuania) and 1 U of *Sau*3A1 (↓GATC; Roche Molecular Biochemicals, Indianapolis, USA) at 37ºC.

Products showing different sizes or different digestion profiles were used for subsequent sequence analysis of integron variable regions.

3.2.5 DNA extraction, plasmid isolation and integron genomic location

In order to determine the location of integrons, total genomic DNA and plasmid DNA were extracted and purified using the Genomic DNA Purification Kit (MBI Fermentas, Vilnius, Lithuania) and the EZNA Plasmid Mini Kit II (Omega Bio-tek, Georgia, USA), respectively, according to the manufacturer's instructions. Aliquots were loaded onto 0.8% agarose gels and separated by electrophoresis at 80 V for 75 min. Gels were then stained and documented as described above. DNA was transferred to a nylon membrane (Hybond N+; Amersham, Freiburg, Germany) and hybridized with integrase probes, as described above.

Table 3.1 Oligonucleotides used in this study.

Prime Pair	Target	Sequence (5'-3')	Anneling temperature (ºC)	Amplicon size (bp)	Reference
intI1F intI1R	*intI1*	CCT CCC GCA CGA TGA TC TCC ACG CAT CGT CAG GC	55	280	Kraft *et al.,* 1986
intI2F intI2R	*intI2*	TTA TTG CTG GGA TTA GGC ACG GCT ACC CTC TGT TAT C	52	233	Goldstein *et al.,* 2001
intI3F intI3R	*intI3*	AGT GGG TGG CGA ATG AGT G TGT TCT TGT ATC GGC AGG TG	50	600	Goldstein *et al.,* 2001
5'-CS 3'-CS	class 1 integron variable region	GGC ATC CAA GCA GCA AG AAG CAG ACT TGA CCT GA	58.5	variable	Levesque *et al.,* 1995
hep74 hep51	class 2 integron variable region	CGG GAT CCC GGA CGG CAT GCA CGA TTT GTA GATGCCATCGCAAGTACGAG	60	variable	White *et al.,* 2001
REP1R REP2I	REP elements	III ICG ICG ICA TCI GGC NCG ICT TAT CIG GCC TAC	40	variable	Versalovic *et al.,* 1991
27F 1492R	16S *rRNA* gene	AGAGTTTGATCCTGGCTCAG GGYTACCTTGTTAACGACTT	51	1467	Lane, 1991
gyrB3F gyrB14R	*gyrB*	TCCGGCGGTCTGCACGGCGT TTGTCCGGGTTGTACTCGTC	55	1110	Yáñez *et al.,* 2003

3.2.6 Antibiotic susceptibility testing

Integrase-positive isolates were tested for antibiotic susceptibility by using the disk diffusion method according to Clinical and Laboratory Standards Institute recommendations (CLSI, 2005).

The following antimicrobials were tested: ampicillin (AMP, 10 μg), aztreonam (ATM, 30 μg), ceftazidime (CAZ, 30 μg), cefalothin (CEF, 30 μg), ciprofloxacin (CIP, 5 μg), chloramphenicol (CHL, 30 μg), erythromycin (ERY, 15 μg), gentamicin (GEN, 10 μg), imipenem (IPM, 10 μg), nalidixic acid (NAL, 30 μg), streptomycin (STR, 10 μg), tetracycline (TET, 30 μg) and trimethoprim-sulfamethoxazole (STX, 25 μg) (Oxoid, Basingstoke, United Kingdom).

3.2.7 Conjugative gene transfer

Integrase-positive strains containing integron-carrying plasmids (MM.1.3, MM.1.5, MM.2.2, MM.2.11) were included as donors in mating assays. Rifampicin-resistant *E. coli* CV601-GFP was used as recipient strain (Smalla *et al.*, 2006). Overnight cultures of donor and recipient strains were adjusted to an optical density of 0.6 at 600 nm. Recipient and donor strains were mixed (donor to recipient ratio of 1:1) in 0.9% NaCl solution and filtered through 0.45 μm-pore size nitrocellulose filters. Filters were placed on Trypticase Soy Agar (TSA) plates and incubated at 37°C overnight. Cells were washed off the filter by vortexing in 10 mL of 0.9% NaCl. Serial dilutions were prepared and aliquots of 100 μL were spread on TSA plates containing rifampicin (100 $mg.L^{-1}$), kanamycin (30 $mg.L^{-1}$) and streptomycin (50 $mg.L^{-1}$). Assays were run in duplicate. Donor and recipient strains were also placed on the selective plates for mutant detection. Putative transconjugants were confirmed by REP-PCR and amplification of *intI* genes as described above.

3.2.8 Nucleotide sequence accession numbers

Sequences of the 16S rRNA gene and integron gene cassette arrays were deposited in the GenBank public database under the accession numbers EF550539-EF550580 and EU089665-EU089673, respectively.

3.3 Results

3.3.1 Sampling and bacterial isolation

Physico-chemical characterization of samples collected is shown in **Table 3.2**. Final effluent concentrations were in conformity with the Portuguese emission standards (DL 236/98). Waters were filtered through membranes and placed onto selective media for *Aeromonas* and *Enterobacteriaceae*. A total of 286 bacterial isolates were obtained (158 on MacConkey-agar and 128 on GSP-agar) from each of the following treatment stages: raw water (RW, *n*=61), homogeny tank (HT, *n*=41), aeration tank (AT, *n*=35), sludge recirculation (SR, *n*=55) and final effluent (FE, *n*=94). Bacterial isolates were further screened for the presence of integrons.

Table 3.2 Characterization of slaughterhouse wastewater samples at different stages of treatment process (RW, raw water; HT, homogeny tank; AT, aeration tank; SR, sludge recirculation; FE, final effluent) in terms of pH, chemical oxygen demand (COD), biological oxygen demand (BOD_5), total suspended soils (TSS) and oil/grease.

Sampling Site	pH (at 20°C)	COD ($g.L^{-1}$ O_2)	BOD_5 ($g.L^{-1}$ O_2)	TSS ($g.L^{-1}$)	Oil/grease ($mg.L^{-1}$)
RW	6.7	4.1	2.6	2.3	636.1
HT	6.8	3.4	2.7	3.1	488.7
AT	7.1	-	-	7.5	-
SR	7.0	-	-	10.8	-
FE	7.1	68.9	17.7	20	<10

3.3.2 Screening of integrons and phylogenetic affiliation of intI-positive isolates

Figure 3.1 represents the prevalence of integron-positive isolates along the wastewater treatment process.

intI1-carrying bacteria corresponded to 30.7% (88 out of 286) of the isolates and were detected in all stages of the wastewater treatment process, being more abundant in the aeration tank and final effluent. The *intI2* gene was detected in 4.5% (13 out of 286) of bacterial isolates, being present only in raw water, aeration tank and final effluent. No positive detection was obtained for *intI3*. One isolate (MM.2.1, from raw water) possessed simultaneously a class 1 and a class 2 integrase.

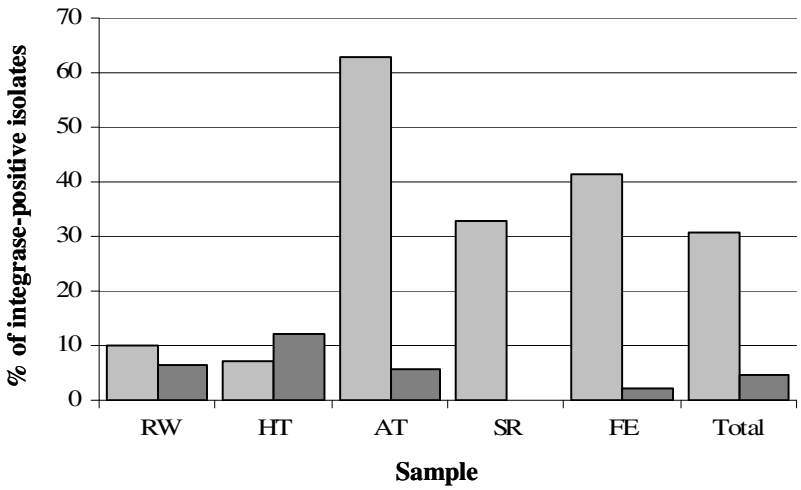


Figure 3.1 Percentage of class 1 (light grey) and class 2 (dark grey) integrase-positive isolates detected in the wastewater treatment plant of Mirandela, Portugal, at different stages of treatment process. Abbreviations: RW, raw water (*n*=61); HT, homogeny tank (*n*=41); AT, aeration tank (*n*=35); SR, sludge recirculation (*n*=55); FE, final effluent (*n*=94); Total, global prevalence of integrase-positive isolates in the WWTP (N=286).

Forty-two different REP-PCR profiles (shown in **Figure 3.2**) were obtained out of 100 integrase-positive isolates. Subsequent sequencing of the 16S rRNA gene allowed to affiliate integrase-positive strains to *Aeromonas* sp. (69%), isolated from all samples except raw water; *Escherichia* sp. (28.6%), isolated from all samples except aeration tank, and *Morganella morganii* (2.4%), only found in raw water (**Table 3.3**).

3.3.3 Detection and characterization of integron-associated gene cassettes

The genetic content of 43 integrons was determined through PCR amplification of integron-associated variable regions. Results obtained are summarized in **Table 3.3.**

PCR products were obtained for 26 *intI1*-positive strains and 5 *intI2*-positive strains, ranging from approximately 1 kb to 2.5 kb. No amplification products were detected in nearly 28% (12 out of 42) of the strains. Digestion of PCR products with restriction enzymes (*Hae*III and *Sau*3A1) allowed the identification of 8 distinct cassette arrays in class 1 integrons and 2 in integrons belonging to class 2 (**Figure 3.3**). As determined by subsequent sequencing analysis, all cassette arrays contained genes coding for aminoglycoside adenyltransferases conferring resistance to streptomycin and spectinomycin (*aadA1*, *aadA2* and *aadA13*), alone or in combination with other resistance genes. Genes encoding dihydrofolate reductases (*dfrA1* and *dfrA12*), conferring resistance to trimethoprim, were also frequently (46.5%) found in *intI*-positive isolates. Cassettes encoding streptothricin acetyltransferases (*sat2*), a β-lactamase (bla_{OXA-1}), chloramphenicol acetyltransferase (*catB3*), a putative esterase (*estX*) and a protein with unknown function (*orfF*) were also detected (**Table 3.3**). Common gene cassettes arrays were found in both *Aeromonas* sp. and *Enterobacteriaceae* (e.g. *dfrA1-aadA1* in class 1 integrons and *estX-sat2-aadA1* in class 2 integrons).

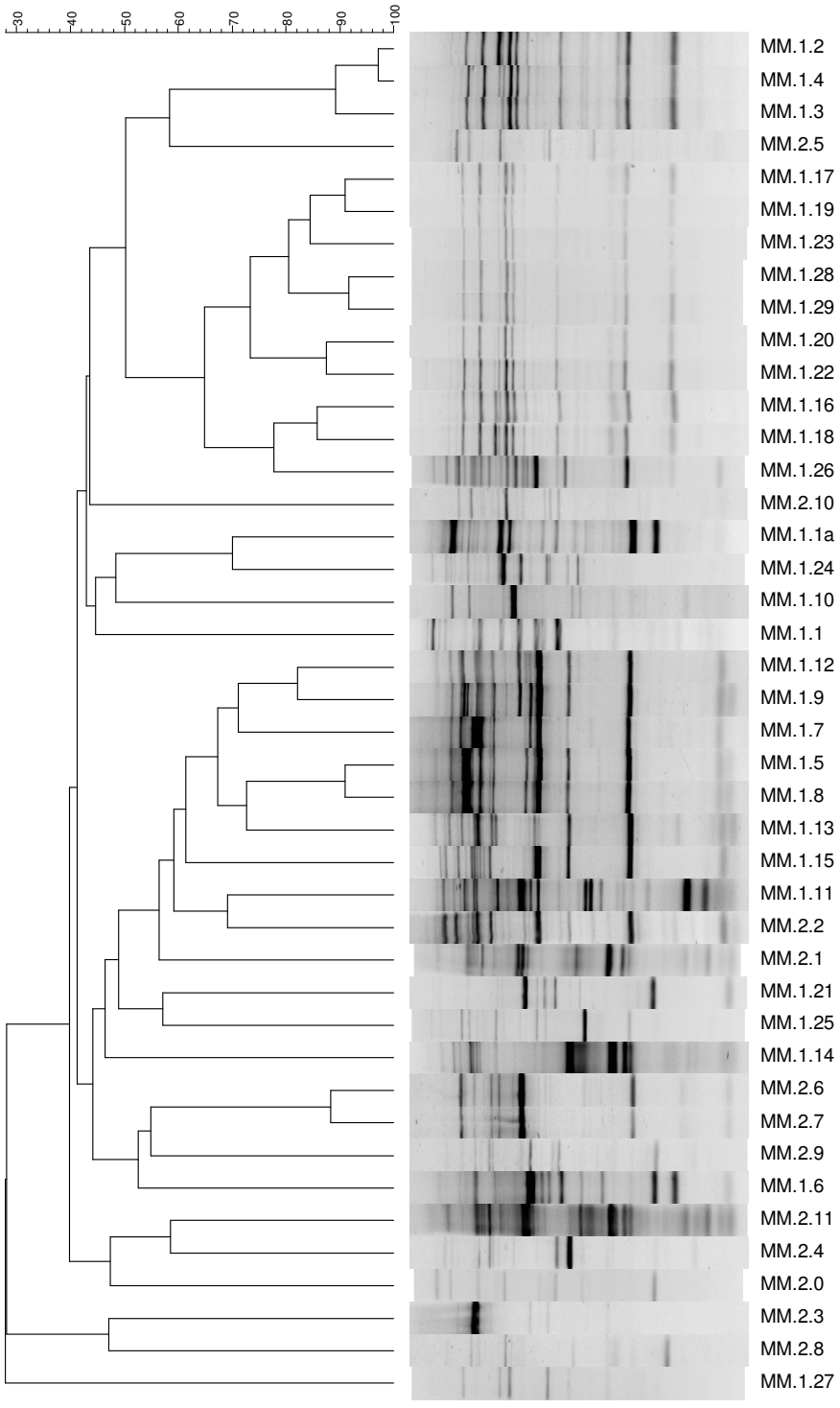


Figure 3.2 UPGMA dendrogram generated from the 42 different REP profiles of *intI*-positive isolates based on Dice similarity correlation.

Table 3.3 Characterization of integrase-positive strains isolated at different stages of the WWTP of Mirandela, Portugal, in terms of dispersion, phylogenetic affiliation, antibiotic resistance (and intermediate) phenotype, gene cassette arrays, structure of 3'-segment and integron location (C, chromosome; P, plasmid). Structures not detected are represented with a single dash.

Strain	Presence at WWTP[a]	Phylogenetic Affilliation	Resistance Phenotype[c]	*intI* gene	Gene Cassette Array[d]	Structure 3' segment	Integron Putative Location[e]	Accession Number
MM.1.11	RW	*Escherichia coli*	ERY, TET, STX (CEF, STR)	I	*dfrA1 - aadA1*	*qacEdelta1*	C	EF550550
MM.1.12	RW	*Escherichia coli*	AMP, ERY, STR, TET (CEF)	I	*bla* $_{OXA-1}$ *- aadA1*	*qacEdelta1-sul1*	C	EF550551
MM.1.13	RW	*Escherichia coli*	AMP, CEF, CHL, ERY, STR, STX, TET	I	*dfrA1 - aadA1*	*qacEdelta1-sul1*	C	EF550552
MM.1.14	RW	*Escherichia coli*	AMP, CHL, ERY, STR, STX, TET (CEF)	I	*dfrA12 - orfF - aadA2*	-	C	EF550553
MM.1.15	RW	*Escherichia coli*	ERY, STR, TET	I	*aadA1*	*sul1*	C	EF550554
MM.2.1	RW	*Escherichia coli*	ERY, STR, STX, TET	I	*dfrA1 - sat1- aadA1*	*qacEdelta1*	C	EF550570
				II	*aadA13 - sat2*	*ybeA*	C	EF550570
MM.2.2	RW	*Escherichia coli*	CEF, ERY, STR, STX, TET	II	*dfrA1 - sat1 - aadA1*	*ybeA*	C, P	EF550571
MM.2.3	RW	*Morganella morganii*	AMP, CEF, ERY, STR, STX, TET	II	*dfrA1 - sat1-aadA1*	*ybeA*	C	EF550572
MM.1.1a	HT	*Aeromonas* sp.	AMP, CEF, CHL, STR, TET (ERY)	I	*aadA2*	*qacEdelta1-sul1*	C	EF550540
MM.1.9	HT	*Escherichia coli*	CEF, ERY, STR, TET	I	*aadA1*	*qacEdelta1-sul1*	C	EF550548
MM.1.10	HT	*Aeromonas veronii*	AMP (ERY, STR, TET)	I	*aadA2*	*qacEdelta1-sul1*	C	EF550549
MM.2.0	HT	*Aeromonas* sp.	AMP, TET (ERY, STR)	II	-	-	C	EF550569
MM.2.4	HT	*Aeromonas media*	AMP, CEF, ERY	II	-	-	C	EF550573
MM.2.5	HT	*Aeromonas* sp.	AMP, CEF, STR (ERY)	II	-	-	C	EF550574
MM.2.6	HT	*Escherichia coli*	ERY (STR)	II	-	-	C, P	EF550575
MM.2.7	HT	*Aeromonas* sp.	AMP, CEF, ERY	II	-	-	C	EF550576
MM.1.8	AT	*Aeromonas* sp.	AMP, CEF, STR, STX, TET (ERY)	I	*dfrA1 - aadA1*	*qacEdelta1-sul1*	C	EF550547
MM.1.16	AT	*Aeromonas salmonicida*	AMP, CEF, STR, STX, TET (ERY)	I	*dfrA1 - aadA1*	*qacEdelta1-sul1*	C	EF550555
MM.1.17	AT	*Aeromonas salmonicida*	AMP, CEF, STR, STX, TET (ERY)	I	*dfrA1 - aadA1*	*qacEdelta1-sul1*	C	EF550556
MM.1.18	AT	*Aeromonas salmonicida*	AMP, CEF, STR, STX (ERY, TET)	I	*dfrA1 - aadA1*	*qacEdelta1-sul1*	C	EF550557
MM.1.19	AT	*Aeromonas salmonicida*	AMP, CEF, STR, STX, TET (ERY)	I	*dfrA1 - aadA1*	*qacEdelta1-sul1*	C	EF550558
MM.1.20	AT	*Aeromonas salmonicida*	AMP, CEF, STR, STX, TET (ERY)	I	*dfrA1 - aadA1*	*qacEdelta1-sul1*	C	EF550559
MM.2.8	AT	*Aeromonas veronii*	AMP, ERY (STR)	II	-	-	C	EF550577
MM.2.9	AT	*Aeromonas media*	AMP, CEF, ERY	II	-	-	C	EF550578
MM.1.2	AT, SR, FE	*Aeromonas salmonicida*	AMP, CEF, STR, STX, TET (ERY)	I	*dfrA1 - aadA1*	*qacEdelta1-sul1*	C	EF550541
MM.1.4	AT, SR, FE	*Aeromonas salmonicida*	AMP, CEF, STR, STX, TET (ERY)	I	*dfrA1 - aadA1*	*qacEdelta1-sul1*	C	EF550543
MM.1.6	SR	*Aeromonas* sp.	AMP, ERY, IPM, STR, TET (CEF, STX)	I	*catB3 - aadA1*	*qacEdelta1-sul1*	C	EF550545
MM.1.7	SR	*Escherichia coli*	CEF, CHL, ERY,GEN, NAL, STR, STX, TET	I	*aadB - aadA1* ::ISUnCu1	*qacEdelta1-sul1*	C	EF550546
MM.1.21	SR	*Aeromonas hydrophila*	AMP, CEF (STR)	I	-	-	C	EF550560
MM.1.22	SR	*Aeromonas salmonicida*	AMP, CEF, STR, STX, TET (ERY)	I	*dfrA1 - aadA1*	*qacEdelta1-sul1*	C	EF550561
MM.1.23	SR	*Aeromonas salmonicida*	AMP, CEF, STR, STX, TET (ERY)	I	*dfrA1 - aadA1*	*qacEdelta1-sul1*	C	EF550562
MM.1.24	SR	*Aeromonas jandaei*	AMP, CEF, ERY, IPM, STR	I	*aadA1*	*qacEdelta1-sul1*	C	EF550563
MM.1.3	SR, FE	*Aeromonas salmonicida*	AMP, CEF, STR, STX (ERY)	I	*dfrA1 - aadA1*	*qacEdelta1-sul1*	C, P	EF550542
MM.1.1	FE	*Aeromonas allosaccharophila*	AMP, ERY, GEN, STR (IPM, NAL)	I	-	-	C	EF550539
MM.1.5	FE	*Escherichia coli*	AMP, CEF, ERY, STR, STX, TET	I	*dfrA1- aadA1*	*qacEdelta1-sul1*	P	EF550544
MM.1.25	FE	*Aeromonas caviae*	AMP, CEF, ERY (STR)	I	-	-	C	EF550564
MM.1.26	FE	*Aeromonas salmonicida*	AMP, CEF, STR, STX, TET (ERY)	I	*dfrA1 - aadA1*	*qacEdelta1-sul1*	C	EF550565
MM.1.27	FE	*Aeromonas veronii*	AMP (ERY)	I	-	-	C	EF550566
MM.1.28	FE	*Aeromonas salmonicida*	AMP, CEF, STR (ERY, TET)	I	-	-	C	EF550567
MM.1.29	FE	*Aeromonas salmonicida*	AMP, CAZ, CHL, CEF, ERY, GEN, IPM, STR, STX, TE	I	*dfrA1 - aadA1*	*qacEdelta1-sul1*	C	EF550568
MM.2.10	FE	*Aeromonas* sp.	AMP, STR (ERY, TET)	II	*estX-sat2-aadA1*	*ybeA*	C	EF550579
MM.2.11	FE	*Escherichia coli*	ERY, STR, TET	II	*estX-sat2-aadA1*	*ybeA*	P	EF550580

In addition, an insertion sequence belonging to IS110 family was detected in *E. coli* MM.1.7 and shared 100% homology with IS*UnCu1* found in IncP-1α plasmid pSp7 isolated from activated sludge (Tennstedt *et al.*, 2003).

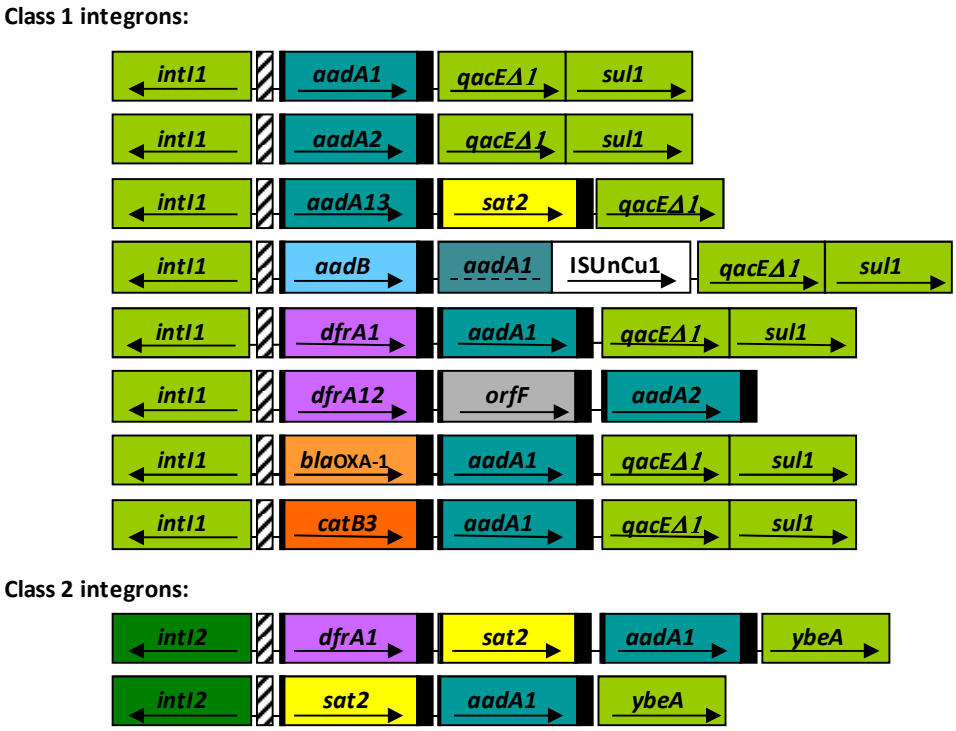


Figure 3.3 Schematic representation of integron structures detected in slaughterhouse wastewaters. Arrows indicate the direction of translation, dashed arrows represent truncated open reading frames, *attI1* sites are represented as striped boxes and *attC* sites as black boxes.

3.3.4 Genomic location of integrons

Most integrons (38) were exclusively located in chromosomal DNA, whereas 2 integrons (in strains MM.1.5 and MM.2.11) were exclusively located in plasmid

DNA. Three strains (MM.1.3, MM.2.2 and MM.2.6) gave positive hybridization signals in both chromosomal and plasmid DNA. In strains MM.1.3 and MM.2.2, amplification of integron variable regions and subsequent digestion with restriction enzymes detected only one type of gene cassette array present, suggesting the presence of at least 2 copies of those integrons.

3.3.5 Conjugative gene transfer

The *intI*-positive plasmids of isolates *Aeromonas* sp. MM.1.3 and *E. coli* MM.1.5, both carrying *dfrA1-aadA1* gene cassette arrays, were successfully transferred into *E. coli* CV601-GFP at transfer frequencies of 3.79×10^{-5} and 5.46×10^{-5} transconjugants/recipient, respectively. No transconjugants were obtained from experiments using *E. coli* MM.2.2 and *E. coli* MM.2.11 as donor strains. The detection limit was 7.59×10^{-7} transconjugants/recipient.

3.3.6 Antibiotic susceptibility testing

The antimicrobial agents used for susceptibility testing were chosen to cover different antibiotic groups and/or based on resistance genes associated with integrons (Fluit & Schmitz, 1999; Nordmann & Poirel, 2005). Nearly 50% of integrase-positive strains (20 out of 42) showed a pattern of multiresistance to 5 or more antibiotics and nearly 81% (34 out of 42) were multiresistant to 3 or more antibiotics of different classes (**Table 3.3**). Among *Aeromonas* sp., the most frequent resistances was to ampicillin (100%), cefalothin (76%), streptomycin (69%), tetracycline (48%) and trimethoprim-sulfamethoxazole (45%), whereas none of the isolates showed resistance to aztreonam, ciprofloxacin or nalidixic acid. Among *Enterobacteriaceae*, there was a higher resistance to erythromycin (100%),

tetracycline (92%) and streptomycin (85%); no isolates were found to be resistant to aztreonam, ceftazidime, ciprofloxacin, imipenem nor nalidixic acid (**Figure 3.4**).

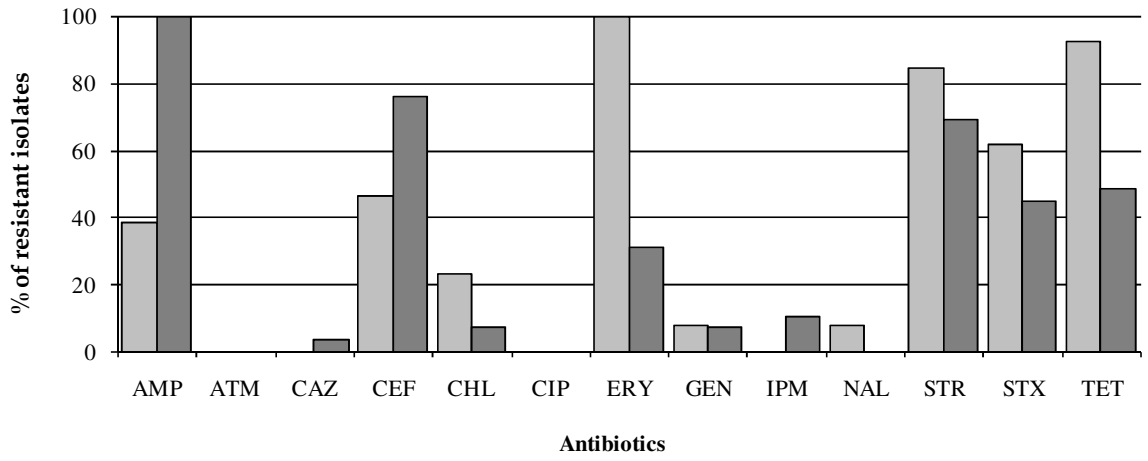


Figure 3.4 Antimicrobial resistance phenotypes of *Enterobacteriaceae* (light grey) and *Aeromonas* (dark grey) integrase-positive isolates. Antibiotic abbreviations: AMP, ampicillin; ATM, aztreonam; CAZ, ceftazidime; CEF, cefalothin; CHL, chloramphenicol; CIP, ciprofloxacin; ERY, erythromycin; GEN, gentamicin; IPM, imipenem; NAL, nalidixic acid; STR, streptomycin; STX, trimethoprim-sulfamethoxazole; TET, tetracycline.

3.4 Discussion

In this study, the prevalence of integrons was investigated in a collection of bacterial isolates from a wastewater treatment plant receiving waters from a slaughterhouse. Two bacterial groups were selected to represent aquatic naturally occurring bacteria (*Aeromonas*) and bacteria mainly introduced due to human activity (*Enterobacteriaceae*).

The prevalence of class 1 and class 2 integrons among *Aeromonas* and *Enterobacteriaceae* isolates in the WWTP of Mirandela was found to be of 30.7% and 4.5%, respectively. *intI3* genes, originally identified in clinical *Serratia marcescens*

and *Klebsiella pneumoniae (*Arakawa *et al.*, 1995; Correia *et al.*, 2003*),* were not detected among the wastewater isolates included in the present study. The global prevalence of integron-carrying bacteria was 35%, a value superior to those usually reported for other types of aquatic environments (Rosser & Young, 1999; Roe *et al.*, 2003; Gaze *et al.*, 2005; Henriques *et al.*, 2006).

Integrase genes were detected in all of the wastewater samples. The relative presence of integrons among *Enterobacteriaceae* and *Aeromonas* was expected to be higher at earlier stages of the treatment process (i.e., in raw water, homogeny tank and the aeration tank), due to higher selective pressures, such as lower pH, higher levels of oil and grease, and higher abundance of microorganisms at these stages (**Table 3.2**). However, this was true only for class 2 integrons. Isolates harbouring class 1 integrons were more prevalent in the final effluent, drawing attention to the risk of wastewaters to act as a proper place for HGT, contributing to the spread of harmful genetic determinants in natural environments.

Integrase-positive bacteria were further characterized in terms of phylogenetic affiliation, gene cassette content and antibiotic susceptibility (**Table 3.3**). No amplification product was obtained in 31% of the integrase-positive isolates, due to the lack of a 3'- conserved region**.** Sixty per cent (26 out of 43) of class 1 and 11.6% (5 out of 43) of class 2 integrons harboured at least one gene cassette. Ten arrays were detected containing 1 to 3 cassettes. From those, 8 distinct arrays were present in integrons of class 1, and 2 arrays were contained in class 2 integrons. Common gene cassettes arrays were detected in both *Aeromonas* sp. and *Enterobacteriaceae* isolates. By comparison to other sequences deposited in databases, gene cassettes encoding resistance to streptomycin and spectinomycin, streptothricin, trimethoprim, chloramphenicol and β-lactams were identified, as well as a putative esterase and an *orf* encoding a protein with unknown function. Although aminoglycosides and trimethoprim were often used in the past years to prevent diarrhoea in calves (Du *et al.*, 2005), their use is now limited. Despite this, the array *dfrA1-aadA1* in class 1 integrons was found along the entire process, being the most common array. Previous studies have reported *aadA*- and *dfr*-like

genes as frequently found gene cassettes both in clinical (Chang *et al.*, 2000; Blahna *et al.*, 2006) and environmental (Henriques *et al.*, 2006; Roe *et al.*, 2003; Jacobs & Chenia, 2007) isolates. Additionally, the *dfrA1-aadA1* array is thought to be considerably stable and that its transfer occurs by the mobilization of the complete integron structure in larger mobile elements, such as transposons or plasmids, rather than individual gene cassettes (Martinez-Freijo *et al.*, 1999).

Most integrase genes were located on bacterial chromosomes. The total prevalence of integrases carried in plasmids (11.9%) was similar to that previously reported in wastewaters (Tennstedt *et al.*, 2003). Nevertheless, the prevalence obtained can be biased due to the fact that plasmid and chromosomal DNA can display the same electrophoretic mobility.

Isolates with a putative location of integrons on plasmids were included as donors in mating assays using *E. coli* CV601-GFP as recipient strain. The *dfrA1-aadA1* gene cassette arrays was successfully transferred using *Aeromonas* sp. MM.1.3 and *E. coli* MM.1.5 as donors, which could explain the high prevalence of this array in the WWTP of Mirandela. No transconjugants were obtained using *E. coli* MM.2.11, harbouring the array *estX-sat2-aadA1*, as donor. Nevertheless, the presence of the *estX-sat2-aadA1* array in *Aeromonas* sp. due to horizontal gene transfer is not excluded. Class 2 integron variable regions were amplified using primers that bind to *attI2* and to *ybeA* gene located within transposon Tn*7 (*White *et al.*, 2001). Therefore, the presence of such integron in *Aeromonas* sp. may have had its origin in a transposition event. To the best of our knowledge, this is the first report of a gene cassette array detected in class 2 integrons from *Aeromonas* sp.

Antimicrobial resistance patterns revealed that 47.6% of the integrase-positive strains were multiresistant to five or more antibiotics. Previously, class 1 and 2 integrons were reported to be present together in 10% of *E. coli* isolates, being most prevalent among strains with resistance profiles to five or more antibiotics (Goldstein *et al.*, 2001). Although 11.9% and 33% of *Enterobacteriaceae* and *Aeromonas* sp., respectively, displayed a multiresistance pattern to 5 or more

antibiotics, only one *E. coli* strain possessed simultaneously *intI1* and *intI2* genes. The gene cassettes found in this study did not explain the totality of resistance phenotypes observed, suggesting the probable existence of other resistance mechanisms encoded in the chromosome and/or in other mobile DNA elements not detected in this study.

Isolates MM.1.10 and MM.1.11 exhibited only reduced susceptibility to aminoglycosides, although they possessed *aadA* gene cassettes in their integron structure. The lack of resistance to these antibiotics may be explained by the presence of weak gene cassette promoters that result in lower transcription efficiencies of gene cassette arrays (Martinez-Freijo *et al.*, 1999; Jové *et al.*, 2010), as discussed in more detail in **Chapter 5**.

The use of selective media clearly limits the diversity of wastewater bacteria obtained and leads consequently to an underestimation of true diversity of integrons present in wastewaters. Nevertheless, this approach provided valuable data regarding the taxonomic affiliation, antibiotic resistance phenotype and gene cassettes arrays of integron-carrying bacteria accessible to cultivation among two important bacterial groups.

Wastewater treatment plants constitute potential hot spots for horizontal gene transfer and may represent a major concern regarding the dissemination of undesirable genetic traits in the environment. The results obtained in this study strongly support this hypothesis. Additionally, this study highlights the need to control antibiotic-resistant bacteria in treated effluents, in order to avoid the risk of spreading of harmful genetic determinants through discharges into aquatic ecosystems.

CHAPTER 4

submitted for publication

Prevalence and Characterization of Integrons from Bacteria Isolated from an Urban Wastewater Treatment Plant

4.1 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 the natural environment and with the reuse of treated water and sludge in agriculture (Pescod, 1992).

Due to antibiotic resistance concern, increasing attention has been given to wastewater treatment plants (WWTPs) since they may act as incubators of undesirable genetic traits to natural waters, soils and, eventually, to the food chain (Baquero *et al.*; 2008; **Chapter 2**). Additionally, due to high loads of organic matter and high selective pressures, WWTP are propitious habitats for horizontal gene transfer events (Baquero *et al.*, 2008; Schlüter *et al.*, 2007b).

Integrons are genetic systems that allow bacteria to capture and express gene cassettes. They typically consist of an *intI* gene encoding an integrase that catalyses the incorporation or excision of gene cassettes by site-specific recombination, a recombination site *attI* and a promoter responsible for the expression of inserted gene cassettes (Cambray *et al.*, 2010). Integrons were first reported in clinical isolates in the 1980s (Hall *et al.*, 1999) and continued to be extensively studied in clinical environments, due to their association with other mobile genetic elements and multiresistance phenotypes (Leverstein-van Hall *et al.*, 2003). However, in the last decade special attention has been given to integrons from natural environments as well in order to gather information on their ecology and diversity, and to understand their role in bacterial adaptive potential.

Increasing evidence that integrons may act as important adaptive systems involved in bacterial SOS responses have been reported (Guerin *et al.*, 2009).

Integron-related sequences are found in a wide diversity of bacterial hosts, most of them (~30%) belonging to γ-Proteobacteria (**Chapter 7**). The wide dispersion of integrons is, in part, a consequence of their association to other mobile genetic elements, which not only increases the genetic traits harboured but also the mobilizing events, contributing to the spreading 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 the previous chapter, the prevalence of integrons was investigated among *Enterobacteriaceae* and *Aeromonas* spp. isolated from a WWTP receiving waters from a slaughterhouse (**Chapter 3**). The presence of integrase-carrying bacteria in slaughterhouse's wastewaters was determined to be 35%, a considerable high value when compared to other aquatic environments; nearly 50% of integrase-positive isolates were resistant to 5 or more antibiotics, drawing, once again, attention for the misuse of antibiotics in veterinary therapeutics and for the role of WWTPs 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.

4.2 Material and methods

4.2.1 Sampling and bacterial isolation

Sampling was performed in November 2007 in an activated sludge plant receiving domestic effluents, located in Ermesinde, North of Portugal. Wastewater treatment includes the following steps: preliminary treatment (mechanical

screening and fat separation), primary treatment (sedimentation) and secondary treatment (activated sludge biological treatment and sedimentation). Sludge produced is dried, stabilized and further used as fertilizer in agriculture.

Water samples were collected in 0.5 L autoclaved bottles at 5 points throughout the WWTP: raw water (RW), primary decantation tank (PD), aeration tank (AT), sludge recirculation (SR) and final effluent (FE). Biochemical oxygen demand (BOD_5), chemical oxygen demand (COD), total suspend 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 ^{o}C and 37 °C. All individual colonies were picked from the following dilutions: 10^{-4} and 10^{-5} in raw water, 10^{-3} and 10^{-7} in primary decantation tank, 10^{-4} and 10^{-5} in aeration tank, 10^{-6} and 10^{-8} in sludge recirculation, 10^{-2} and 10^{-2} in final effluent, for MacConkey and GSP agar, respectively.

4.2.2 Integron screening and identification of integrase-positive isolates

Oligonucleotides used during this study are listed in **Table 4.1**. Bacterial colonies were initially screened for the presence of *intI1*, *intI2* and *intI3* genes by dot-blot hybridization, as described previously (**Chapter 3**). 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 (Yáñez *et al.* 2003). Sequence similarity searches were conducted by using the BLAST software (Altschul *et al.*, 1997) and nucleotide sequences were aligned by CLUSTAL X (Thompson *et al.*, 1997). Phylogenetic trees were constructed by the neighbour-joining method (Saitou & Nei, 1987) using MEGA version 4.0 (Tamura *et al.*, 2007).

4.2.3 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 (**Chapter 3**). To determine the structure of the 3'- segment of class 1 integrons, the presence of *qacE, sul1, sul3*, ISCR1 (*orf513*) and *tniC* (*tniR)* genes was also screened (**Table 4.1**).

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 4.1**) and the Extensor Long PCR Master Mix (ABgene, UK) according to manufacturer's instructions.

To determine the location of integrons, total genomic DNA and plasmid DNA were purified, transferred onto positive-charged membranes and Southern-blots were probed using *intI*-digoxigenin-labelled fragments, as described previously (**Chapter 3**).

Table 4.1 Oligonucleotides used in this study.

Primer Name	Sequence (5'-3')	Reference
REP1R	III ICG ICG ICA TCI GGC	Versalovic *et al.*, 1991
REP2I	NCG ICT TAT CIG GCC TAC	Versalovic *et al.*, 1991
27F	AGA GTT TGA TCC TGG CTC AG	Lane, 1991
1492R	GGY TAC CTT GTT AAC GAC TT	Lane, 1991
gyrB3F	TCC GGC GGT CTG CAC GGC GT	Yáñez *et al.*, 2003
gyrB14R	TTG TCC GGG TTG TAC TCG TC	Yáñez *et al.*, 2003
intI1_894F (ER.1.16F)	CCA GTG GAC ATA AGC CTG	This study
intI1F	CCT CCC GCA CGA TGA TC	Kraft *et al.*, 1986
intI1R	TCC ACG CAT CGT CAG GC	Kraft *et al.*, 1986
intI2F	TTA TTG CTG GGA TTA GGC	Goldstein *et al.*, 2001
intI2R	ACG GCT ACC CTC TGT TAT C	Goldstein *et al.*, 2001
intI3F	AGT GGG TGG CGA ATG AGT G	Goldstein *et al.*, 2001
intI3R	TGT TCT TGT ATC GGC AGG TG	Goldstein *et al.*, 2001
5'-CS	GGC ATC CAA GCA GCA AG	Levesque *et al.*, 1995
3'-CS	AAG CAG ACT TGA CCT GA	Levesque *et al.*, 1995
hep74	CGG GAT CCC GGA CGG CAT GCA CGA TTT GTA	White *et al.*, 2001
hep51	GAT GCC ATC GCA AGT ACG AG	White *et al.*, 2001
qacE-F	ATC GCA ATA GTT GGC GAA GT	Sandvang *et al.*, 1997
qacE-R	CAA GCT TTT GCC CAT GAA GC	Sandvang *et al.*, 1997
qacE-R (ER.1.4R)	CGG TGT TGC TTA TGC AGT C	This study
sulF1	CTG AAC GAT ATC CAA GGA TTY CC	Heuer & Smalla, 2007
sulR1	AAA AAT CCC ATC CCC GGR TC	Heuer & Smalla, 2007
sul3F	AAG AAG CCC ATA CCC GGR TC	Heuer & Smalla, 2007
sul3R	ATT AAT GAT ATT CAA GGT TTY CC	Heuer & Smalla, 2007
orf513_6F	ATG GTT TCA TGC GGG TT	Arduíno *et al.*, 2003
orf513_7R	CTG AGG GTG TGA GCG AG	Arduíno *et al.*, 2003
RH506 (tniR)	TTC AGC CGC ATA AAT GGA G	Post *et al.*, 2007
tniCF	CGA TCT CTG CGA AGA ACT CG	Toleman *et al.*, 2007
tnsE_R (23CS)	TGG GCT GAG AGA GTG GT	Ramírez *et al.*, 2005
OXA-A_R	AGT GTG TTT AGA ATG GTG ATC	Ouellette *et al.*, 1997
OXA-B_F	CAA GCC AAA GGC ACG ATA GTT G	Henriques *et al.*, 2006
OXA-B_R	CTC AAC CCA TCC TAC CCA CC	Henriques *et al.*, 2006
oxa-2_805Fwd	GTC AAC TCG GAC GCT GC	This study
oxa-2_22Rev	TCG CGA AGA TTC GGA TTG C	This study
oxa-10R (ER.1.17R)	GAC GAT GAA AAT GTT TCG TGC	This study
catB3F	GAC CAA CTA CTT TGA TAG CC	This study
catB3R	TAG CGG GAT TGC CGC CAA CG	Santos *et al.*, 2009
aadA1F	ATG AGG GAA GCG GTG ATC GC	Santos *et al.*, 2010
aadA1R	GCC ACG GAA TGA TGT CGT CG	Santos *et al.*, 2010
aadA13R (ER.1.7R)	TCA CTG AGT CCC TCA TG	This study
aacA4F (ER.1.17F)	CGA GCG AAC ACG CAG TG	This study
aacA4F (ER.1.4F)	CTG GGC AAA GGC TTG GGA A	This study
arr3R (ER.1.4R)	CAG TGA CAT AGC AAG TTC AG	This study
ISAs12_F (ER.1.7F)	GCG CAA ACT CAT CCT GC	This study

4.2.4 Conjugative gene transfer

Integrase-positive strains containing integron-carrying plasmids were used as donors in mating assays using rifampicin- and kanamycin-resistant *Escherichia coli* CV601-GFP as recipient strain (Smalla *et al.*, 2006). Transconjugants were selected on Trypticase Soy Agar (TSA) plates containing rifampicin (100 $mg.L^{-1}$), kanamycin (30 $mg.L^{-1}$) and streptomycin (50 $mg.L^{-1}$) and confirmed by REP-PCR and amplification of integrase gene, as previously described (**Chapter 3**).

4.2.5 Antibiotic susceptibility testing

Integrase-positive isolates were tested for antibiotic susceptibility by using the disk diffusion method according to the Clinical and Laboratory Standards Institute guidelines (CLSI, 2005). *Escherichia coli* ATTC 29522 was used as control strain.

The following antimicrobials were tested: ampicillin (AMP, 10 μg), aztreonam (ATM, 30 μg), ceftazidime (CAZ, 30 μg), cefalothin (CEF, 30 μg), ciprofloxacin (CIP, 5 μg), chloramphenicol (CHL, 30 μg), erythromycin (ERY, 15 μg), gentamicin (GEN, 10 μg), imipenem (IPM, 10 μg), kanamycin (KAN, 30 μg), nalidixic acid (NAL, 30 μg), streptomycin (STR, 10 μg), tetracycline (TET, 30 μg) and trimethoprim/sulfamethoxazole (STX, 25 μg) (Oxoid, Basingstoke, United Kingdom).

4.2.6 Nucleotide sequence accession numbers

Nucleotide sequences were deposited in GenBank under the accession numbers: FJ464567-FJ464590 (16S rRNA gene), FJ238489-FJ238507 (*gyrB*), FJ460175-FJ460183 and HQ170510-HQ170518 (gene cassette arrays).

4.3 Results

4.3.1 Sampling and bacterial isolation

Physico-chemical characterization of samples collected is shown in **Table 4.2**. Final effluent concentrations were in conformity with Portuguese emission standards of water quality (DL 236/98).

A total of 697 bacterial isolates were obtained (292 on MacConkey-agar and 405 on GSP-agar) from each of the following treatment stages: raw waters (RW, *n*=95), primary decantation tank (PD, *n*=193), aeration tank (AT, *n*=169), sludge recirculation (SR, *n*=109) and final effluent (FE, *n*=131).

Table 4.2 Characterization of urban wastewater samples at different stages of treatment process (RW, raw water; PD, primary decantation tank; AT, aeration tank; SR, sludge recirculation; FE, final effluent) in terms of pH, chemical oxygen demand (COD), biological oxygen demand (BOD_5) and, total suspended soils (TSS).

Sampling Site	pH (at 20°C)	COD ($g.L^{-1}$ O_2)	BOD5 ($g.L^{-1}$ O_2)	TSS ($g.L^{-1}$)
RW	7.3	2.9	0.4	0.3
PD	7.2	2.8	0.4	0.2
AT	7.1	-	-	3.2
SR	7.1	-	-	3.6
FE	7.6	0.13	0.04	0.9

4.3.2 Prevalence of integrase genes in wastewater samples

The prevalence of *intI* genes along the WWTP of Ermesinde is shown in **Figure 4.1**. Positive *intI*-hybridization signals were obtained in 3.73% (26 out of 697) bacterial colonies. Integrase genes were detected in all samples except in sludge recirculation. Prevalence of *intI1* was 3.59% (25 out of 697), being more

abundant in the raw waters. Genes encoding IntI2 were present in 0.14% (1 out of 697), being detected only in aeration tank. No *intI3* genes were detected.

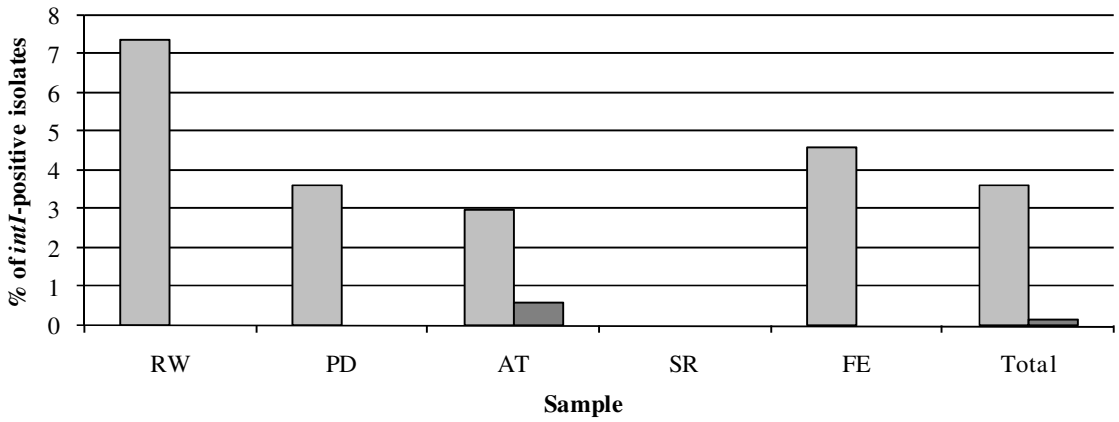


Figure 4.1 Percentage of class 1 (light grey) and class 2 (dark grey) integrase-positive isolates detected in the wastewater treatment plant of Ermesinde, Portugal, at different stages of treatment process. Abbreviations: RW, raw waters (*n*=95); PD, primary decantation tank (*n*=193); AT, aeration tank (*n*=169); SR, sludge recirculation (*n*=109); FE, final effluent (*n*=131); Total, global prevalence of integrase-positive isolates in the WWTP (N=697).

4.3.3 Identification of integrase-positive bacteria

After isolation of *intI*-positive colonies and molecular typing, 24 isolates out of 26 displayed different REP-profiles and were further characterized (**Figure 4.2**). On the basis of 16S rRNA gene, 79.2% (19/24) of *intI*-positive strains were affiliated to the genus *Aeromonas* and 20.8% (5/24) belonged to *Enterobacteriaceae*.

Class 1 integrons were found in *A. allosaccharophila, A. caviae, A. media, A. salmonicida, A. veronii, Enterobacter cloacae, Klebsiella oxytoca, Kluyvera cryocrescens and Shigella* sp. Class 2 integrons were only detected in one *Enterobacter* sp. isolate (**Table 4.3**).

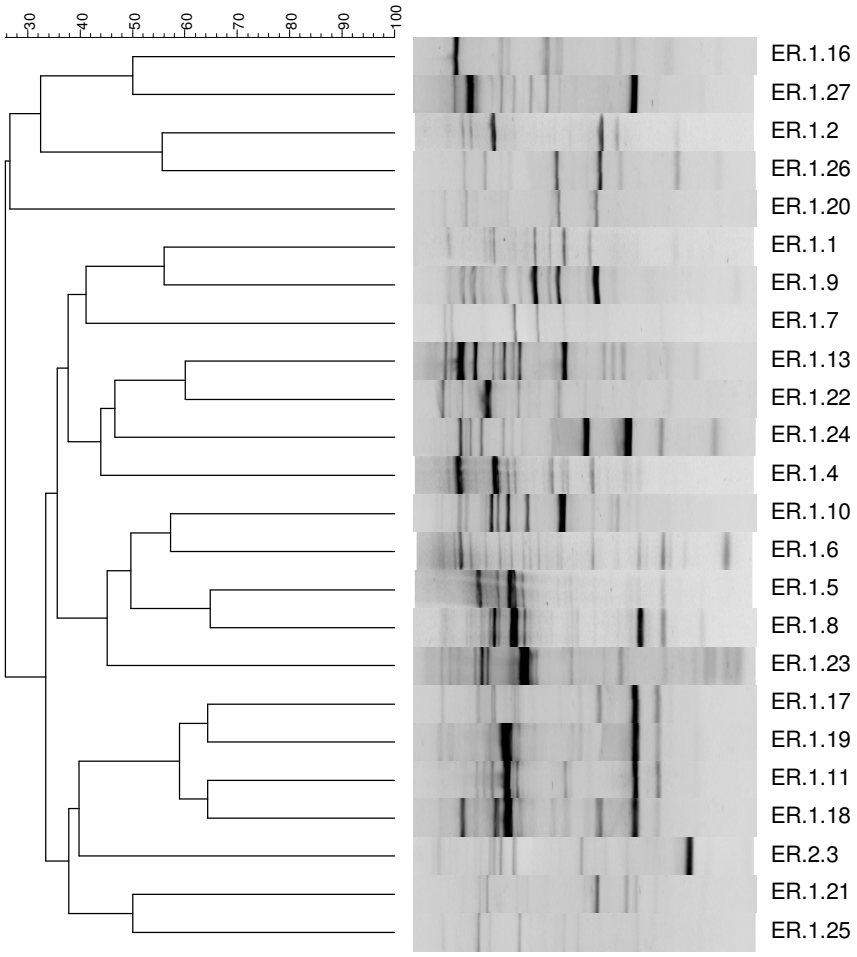


Figure 4.2 UPGMA dendrogram generated from 24 different REP profiles of *intI*-positive isolates based on Dice similarity correlation.

4.3.4 Integron characterization

In 24 strains, 25 integrons were detected; 68% (17 out of 25) of integrons possessed a gene cassette array varying from 0.5 kb to 4 kb. Twelve percent (3 out of 25) produced fragments of 0.15 kb, corresponding to empty integrons, i.e. integrons with no gene cassettes inserted. The remaining 20% (5/25) failed to amplify integron variable region by PCR.

In total, twenty-one different gene cassettes were found organized into 13 distinct arrays, including 4 new arrays (**Figure 4.3**). None of the array sequences determined was common to both *Aeromonas* and *Enterobacteriaceae* (**Table 4.3**). The majority of gene cassettes were involved in antibiotic resistance and included: *aadA*-type genes (resistance to streptomycin and spectinomycin), *aacA4* genes (resistance to amikacin and tobramycin), *aacA4-CR* variants (resistance to amikacin and tobramicin and to fluoroquinolones), *dfrA*-type genes (resistance to trimethoprim), *catB* (resistance to chloramphenicol), *arr*3 (resistance to rifampicin) and extended spectrum β-lactamases bla_{GES} (resistance to penicillins and cephalosporines) and bla_{OXA}-type genes (resistance to penicillins, cephalosporines and oxacillins).

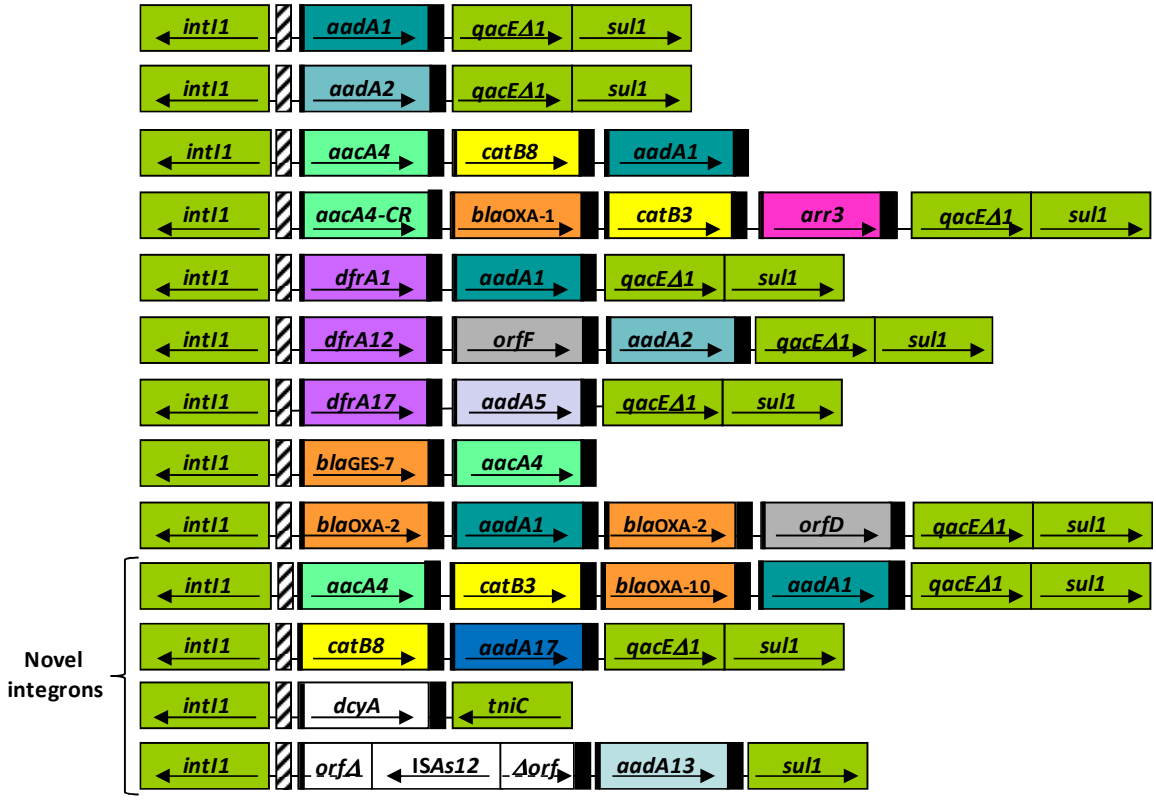


Figure 4.3 Schematic representation of the integron structures detected in this study. Arrows indicate the direction of translation, dashed arrows represent truncated open reading frames, *attI1* sites are represented as striped boxes and *attC* sites as black boxes.

New gene cassettes were also detected (**Figure 4.3**; **Table 4.3**).

A novel *aadA*-variant (named *aadA17*) was found downstream a *catB8* gene cassette, differing from *aadA12* encoding protein by six amino acid substitutions: Pro(26)Ser, His(45)Tyr, Phe(33)Leu, Arg(47)Asn, Lys(51)Thr, Gln(56)Pro (**Figure 4.4**).

In *A. caviae* strain ER.1.20, a novel gene cassette containing an open reading frame of 1083 bp and an *attC* site of 75 bp was detected. The gene cassette was tentatively designated *dcyA* (diguanylate cyclase A) and encodes a putative protein of 360 aa. The putative protein DcyA consists of a Globin-Coupled-Sensor (GCS) domain at the amino terminus and a Diguanylate Cyclase (DGC) domain at the carboxyl terminus and showed 65% identity (BLASTP Expect = $9e^{-135}$) with a diguanylate cyclase from *Shewanella baltica* (accession number YP_001364250). The *dcyA* gene cassette was inserted upstream a *tniC* gene encoding the Tn*402*-resolvase.

```
AADA17          MRVAVTIEISNQLSEVLSVIERHLEPTLLAVHLYGSAVDGGLKPHSDIDLLVTVTVRLDE 60
AADA1           --E--IA-V-T-----VG---------------------------------------- 60
AADA12          -------------------------S------------------Y--------------- 60
AADA15          --E--IA-V-T-----VG---------------------------------------- 60

AADA17          TTRRALINDLLETSASPGESEILRAVEVTIVVHDDIIPWRYPAKRELQFGEWQRNDILAG 120
AADA1           ------------------------------------------------------------ 120
AADA12          ------------------------------------------------------------ 120
AADA15          ------------------------------------------------------------ 120

AADA17          IFEPATIDIDLAILLTKAREHSVALVGPAAEEFFDPVPEQDLFEALRETLKLWNSQPDWA 180
AADA1           --------------------------------L-------------N---T----P---- 180
AADA12          --------------------------------L-------------N---T----P---- 180
AADA15          --------------------------------L-------------N---T----P---- 180

AADA17          GDERNVVLTLSRIWYSAITGKIAPKDVAADWAIKRLPAQYQPVLLEAKQAYLGQKEDHLA 240
AADA1           ----------------V--------------ME---------I---R------E--R-- 240
AADA12          ------------------------------------------------------------ 240
AADA15          ------------------------------------------------------------ 240

AADA17          SRADHLEEFIRFVKGEIIKSVGK 263
AADA1           ----Q----VHY-----T-V--- 263
AADA12          ----------------------- 263
AADA15          ----------------------- 263
```

Figure 4.4 Comparison of the deduced amino acid sequences of the *aadA17* product (AADA17) with its closest relative proteins of the aminoglycoside 3''-adenylyltransferase family: AADA1 (Y18050), AADA12 (AY940491) and AADA15 (DQ393783). Dashes represent conserved amino acids.

Table 4.3 Characterization of *intI*-positive strains isolated at different stages of the WWTP of Ermesinde, Portugal, in terms of dispersion, phylogenetic affiliation, antibiotic resistance phenotype (and intermediate susceptibility in parenthesis), integron structure and putative location (C, chromosomal; P, plasmid). Structures not detected are represented with a single dash.

Strain	Presence at WWTP	Phylogenetic Affilliation	Resistance Phenotype	*intI* gene	Gene Cassette Array	Structure 3' segment	Integron Putative Location	Accession Number
ER.1.1	RW	*Aeromonas media*	AMP, CEF, ERY, KAN, NAL, STR, STX	I	*dfrA12 - orfF - aadA2*	*qacEdelta1-sul1*	C, P	FJ460175
ER.1.2	RW	*Aeromonas caviae*	AMP, CAZ, CEF, ERY, KAN, NAL, STR (CHL, CIP)	I	empty integron	*qacEdelta1-sul1*	C	-
ER.1.4	RW	*Aeromonas allosaccharophila*	AMP, CEF, ERY, GEN, KAN, NAL, STR (CIP)	I	*aacA4-CR - blaOXA-1 - catB3 - arr3*	*qacEdelta1-sul1*	C, P	HQ170510
ER.1.5	RW	*Aeromonas media*	AMP, CEF, ERY, KAN, NAL, STR, STX (CHL)	I	*aadA1*	*qacEdelta1-sul1*	C	FJ460176
ER.1.6	RW	*Aeromonas allosaccharophila*	AMP, CEF, NAL, STR, STX (IPM, ERY)	I	*dfrA1 - aadA1*	*qacEdelta1-sul1*	C	FJ460177
ER.1.7	RW	*Aeromonas salmonicida*	AMP, CEF, NAL, STR	I	*orfER.1.7::*IS*As12 - aadA13*	*sul1*	C	HQ170513
ER.1.8	RW	*Aeromonas media*	AMP, CEF, KAN, NAL, STR (CAZ, GEN)	I	*blaGES-7 - aacA4*	-	C	HQ170511
ER.1.21	PD	*Aeromonas* sp.	AMP, CAZ, CEF, ERY, NAL	I	-	-	C	-
ER.1.22	PD	*Aeromonas media*	AMP, CEF, CHL, CIP, ERY, NAL, TET (STR, KAN)	I	empty integron	*qacEdelta1-sul1*	C, P	-
ER.1.23	PD	*Shigella* sp.	AMP, CEF, CIP, ERY, NAL, STR, STX, TET	I	*dfrA17 – aadA5*	*qacEdelta1-sul1*	C, P	FJ460182
ER.1.24	PD	*Aeromonas veronii*	AMP, CEF, ERY, KAN, NAL, STR	I	*aadA1*	*qacEdelta1-sul1*	C	FJ460183
ER.1.25	PD	*Aeromonas media*	AMP, CEF, CIP, ERY, NAL	I	*aacA4-CR - blaOXA-1 - catB3 - arr3*	*qacEdelta1-sul1*	C	HQ170516
ER.1.26	PD	*Aeromonas caviae*	AMP, CEF, NAL, STR (ERY)	I	*blaOXA-2 - aadA1 - blaOXA-2 - orfD*	*qacEdelta1-sul1*	C	HQ170515
ER.1.9	AT	*Aeromonas caviae*	ATM, CAZ, NAL (STR)	I	*aadA2*	*qacEdelta1-sul1*	C	FJ460178
ER.1.10	AT	*Enterobacter cloacae*	AMP, CEF, ERY (STR)	I	-	-	C, P	-
ER.1.11	AT	*Aeromonas media*	AMP, CEF, NAL, STR (ATM, STX, ERY)	I	*aadA2*	*qacEdelta1-sul1*	C	FJ460179
ER.1.13	AT	*Klebsiella oxytoca*	AMP, ERY, NAL, STR	I	-	-	C	-
ER.2.3	AT	*Enterobacter* sp.	AMP, CEF, ERY	II	-	-	P	-
ER.1.16	FE	*Aeromonas allosaccharophila*	AMP, CEF, ERY, GEN, KAN, NAL, STR, TET	I	*aacA4-CR - blaOXA-1 - catB3 - arr3*	*qacEdelta1-sul1*	C	HQ170517
				I	*catB8 - aadA1*	-	C	HQ170518
ER.1.17	FE	*Aeromonas media*	AMP, CEF, KAN, NAL, STR, TET (STX, ERY)	I	*aacA4 - catB3 - blaOXA10 - aadA1*	*qacEdelta1-sul1*	C	HQ170514
ER.1.18	FE	*Aeromonas media*	AMP, CAZ, CEF, NAL, STR	I	*catB8 – aadA17*	*qacEdelta1-sul1*	C	FJ460181
ER.1.19	FE	*Aeromonas media*	AMP, CEF, NAL	I	-	-	C	-
ER.1.20	FE	*Aeromonas caviae*	AMP, CEF, NAL	I	*dcyA*	*tniC*	C, P	HQ170512
ER.1.27	FE	*Kluyvera cryocrescens*	AMP, CEF, CIP, ERY, NAL	I	empty integron	*qacEdelta1-sul1*	C, P	-

Another new gene cassette (designated *orfER.1.7*) was found in *A. salmonicida* ER.1.7, and consisted of an open reading frame with 660 bp (219 aa) and an *attC* site of 75 bp. The deduced amino acid sequence showed only low similarity with other protein sequences deposited in the GenBank protein database. The closest match was to a hypothetical *Desulfomicrobium baculatum* protein (accession no. YP_003157284) sharing 28% of amino acid identity (BLASTP Expect = $7e^{-11}$). The gene cassette *orfER.1.7* was disrupted by a novel type of insertion sequence (IS) element. The IS was 1460 bp long and belonged to IS*As1* family. It was designated IS*As12*, according to ISFinder nomenclature (www-is.biotoul.fr; Siguier *et al.*, 2006). IS*As12* (GenBank accession no. HQ170513) was flanked by 26 bp imperfect inverted repeats and flanked by a directly repeated 12 bp target sequence. IS*As12* contained an open reading frame of 1161 bp and it is predicted to encode a putative transposase of 386 aa, sharing 94% of identity with a transposase found in *Klebsiella pneumoniae* plasmid pKpQIL_p018 (**Figure 4.5**).

```
ISAs12              MLLFRSALMSIDAVFAQFFDNIHDPRQHAKICYPFYDVLFLTVCAVIGGAEGWEDIEDFG 60
ISKp (ADE43965)     ▒▒▒▒▒▒▒------------D-P--------Y------------------D--------- 52

ISAs12              EVHLPWFQSKGLFKNGIPVHDTIARIISGIKPEPFQAAFVRWTQAINQHTDGALVAIDGK 120
ISKp (ADE43965)     ----------------------------------------------------E--------- 112

ISAs12              TLRSSYDREDRTSTIHMVSAYAAANKLVLGQIKTDAKSNEITAIPELPALLDIKGCLVSI 180
ISKp (ADE43965)     ------N--------------------------------------D-L------------ 172

ISAs12              DAMGCQTEIAETILQQGGDYLLAVKGNQPKLFEAVRQALAPLAATPLCAETMTLEKAHGR 240
ISKp (ADE43965)     ----------------S-------------------------------A----D----- 232

ISAs12              IDGREYHVMAAGELAAQFPHWKQLHSIGVAISYRVENMQKITIEQRYFISSKALVQDEFA 300
ISKp (ADE43965)     -------I-------------E--R---------I-KRE--------Y------TR---- 292

ISAs12              RAVRGHWAIENSLHWVLDVTMGEDDCPIYRGDAAEILACIRHMGLNMLRAETSRKASVRR 360
ISKp (ADE43965)     -S-----------------------------------------------------I-- 352

ISAs12              KQKIAGMSSEYLETVLNAGLKK▒▒▒▒▒▒▒▒▒▒▒▒LGEI▒▒▒ 386
ISKp (ADE43965)     ------------K---G-VSGNPRKFRHSALWNF--GWSAA 394
```

Figure 4.5 Comparison of the deduced amino acid sequences of the transposase IS*As12* with its closest relative transposase from *Klebsiella pneumoniae* plasmid pKpQIL_p018 (accession no. ADE43965). Dashes represent conserved amino acids; gaps are represented as spaces.

4.3.5 Genomic location of integrons

Genomic location of *intI* genes revealed that 16 were exclusively located in chromosomal DNA, while 1 (in *Enterobacter cloacae* ER.2.3) was located exclusively in plasmid DNA. Seven strains (ER.1.1 and ER.1.4, from RW; ER.1.22 and ER.1.23, from PD; ER.1.10, from AT; ER.1.20 and ER.1.27, from FE) possessed *intI* genes simultaneously located in the plasmid and in chromosome (**Table 4.3**).

4.3.6 Conjugative gene transfer

Aeromonas media ER.1.1 and *Shigella* sp. ER.1.23 were used as donors in mating assays; *intI1* genes were successfully transferred at frequencies of $1.47x10^{-5}$ and $1.59x10^{-6}$ transconjugants per recipient cell, respectively.

4.3.7 Antibiotic susceptibility testing

Antimicrobial resistance phenotypes observed are shown in **Figure 4.6**. Nearly 63% of integrase-positive strains (15 out of 24) showed a pattern of multiresistance to 5 or more antibiotics and nearly 80% (19 out of 24) were multiresistant to 3 or more antibiotics of different classes. Among *Enterobacteriaceae,* the most frequent resistances observed were to ampicillin (100%), erythromycin (100%), cefalothin (80%) and nalidixic acid (60%). None showed a resistance profile to aztreonam, chloramphenicol, gentamicin, ceftazidime, imipenem, and kanamycin. Among *Aeromonas* resistances, higher resistances observed were to nalidixic acid (100%), ampicillin (95%), cefalothin (95%), and streptomycin (74%); all strains exhibit susceptibility to imipenem.

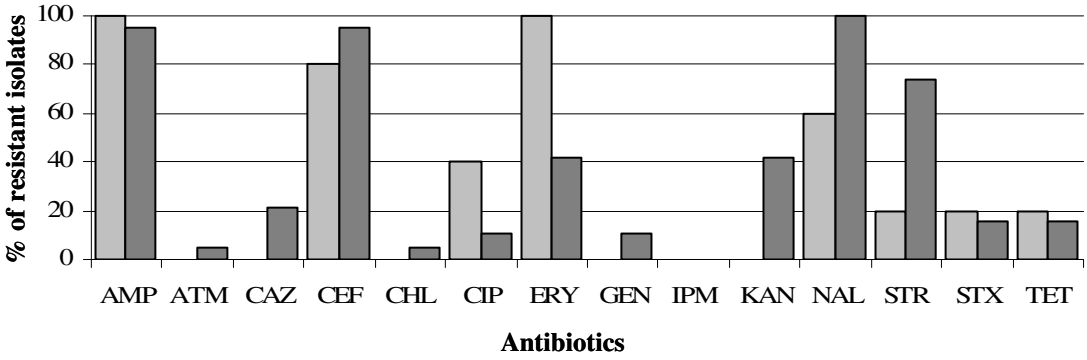


Figure 4.6 Antimicrobial resistance phenotypes of *Enterobacteriaceae* (light grey; *n*=5) and *Aeromonas* (dark grey; *n*=19) integrase-positive isolates. Antibiotic abbreviations: AMP, ampicillin; ATM, aztreonam; CAZ, ceftazidime; CEF, cefalothin; CHL, chloramphenicol; CIP, ciprofloxacin; ERY, erythromycin; GEN, gentamicin; IPM, imipenem; KAN, kanamycin; NAL, nalidixic acid; STR, streptomycin; STX, trimethoprim-sulfamethoxazole; TET, tetracycline.

4.4 Discussion

The reuse of treated effluents and sludge is a common practice in agriculture (Pescod, 1992). However, it has been shown that antibiotics (Xu *et al.*, 2007) and antibiotic-resistant bacteria (**Chapter 3**) still persist in concerning amounts in treated effluents and, therefore, the occurrence of mobile genetic elements in wastewaters should continue to be investigated in order to avoid the perpetuation and dissemination of resistance and/or virulence determinants in soils and aquatic receptacles.

In this study, the prevalence of integrons was assessed in a set of bacterial isolates from an urban WWTP. We focused on two bacterial groups often associated with mobile genetic elements: aeromonads, ubiquitous in aquatic environments and enterobacteria, common inhabitants of polluted waters (Goñi-Urriza *et al.*, 2002).

Surprisingly, the prevalence found (3.73%) was much lower than that previously observed, using the same methodology, in a WWTP receiving effluents

from a slaughterhouse (**Chapter 3**), suggesting that the type of effluent may exert great influence on the occurrence of integrons. The majority of integrase-positive bacteria (~80%) belonged to the genus *Aeromonas,* emphasizing the importance of these natural water-borne bacteria as potential vectors of dissemination of integrons and antibiotic resistance determinants.

In order to evaluate whether the diversity of integron's variable regions was also affected by the type of effluent, molecular characterization of integrons was performed. Contrarily to previous observations on slaughterhouse's effluents (**Chapter 3**), none of the arrays detected were common to both *Aeromonas* and *Enterobacteriaceae*. Nevertheless, higher abundance of gene cassette combinations were found than those reported in slaughterhouse's effluent. This increased diversity of integron structures may be related to a higher diversity of bacterial hosts inhabiting these wastewaters. As observed in the previous study based on culture-independent techniques (**Chapter 2**), this urban effluent was reported as having higher bacterial community diversity, contrarily to slaughterhouse wastewater.

The most frequent gene cassettes detected were *aadA*-type genes, conferring resistance to streptomycin and spectinomycin. In fact, *aadA* are the most often reported gene cassette (http://integrall.bio.ua.pt, **Chapter 7**) in bacterial isolates and regardless of the limited use of such antibiotics nowadays, these gene cassettes seem to persist in the environment.

In this study, a new *aadA*-variant (named *aadA17*) was detected in *A. media* ER.1.18 downstream a *catB8* gene cassette that encodes a chloramphenicol acetyltransferase. Strain ER.1.18 did not exhibit resistance to chloramphenicol, although resistance to streptomycin was observed. Sequence analysis detected the presence of a weak hybrid PcH1 promoter responsible for the expression of *catB-aadA17* cassette array (Jové *et al*., 2010; **Chapter 5**). The deduced CatB8 protein differs from those previously reported by one conserved amino acid substitution, Gln(160)His. Although Gln and His possess similar physical-chemical properties (Frappat *et al*., 2002), this substitution may affect enzyme efficiency, leading to

chloramphenicol susceptibility. However, further investigation is needed to clarify this issue.

A novel gene cassette (designated *dcyA*) was also detected in *Aeromonas caviae* ER.1.20. The putative protein DcyA consists of two domains: a Globin-Coupled-Sensor (GCS) domain and a Diguanylate Cyclase (DGC). GCS domains are predominantly present in Gram-negative α-Proteobacteria, but also in the Firmicutes and Archaea (Freitas *et al.*, 2003). GCS domains detect fluctuations in intracellular concentrations of oxygen, carbon monoxyde, or nitrous oxide and induce signal cascades as specific adaptive responses, which result in aerotaxis and/or gene regulation (Thijs *et al.*, 2007). DGC domains (initially described as domains of unknown function 1, DUF1) are widely present in bacteria and are thought to constitute a general regulatory principle in bacterial growth and development (Jenal, 2004). DGC domains have also been implicated in cell signalling, regulation of bacterial cellular adhesiveness and biofilm formation (Jenal, 2004). It has been discussed that environmental stress can induce the formation of biofilms (O'Toole & Stuart, 2005). Together, these findings are in agreement with those recently reported that place integrons as important players in SOS response (Guerin *et al.*, 2009).

Gene cassettes possessing no putative conserved domains, and thus with unknown function, were also detected. Although antibiotic resistance genes account for the larger majority of gene cassettes reported in bacterial isolates (**Chapter 7**), especially in class 1 integrons, recent studies focusing on genetic libraries of total environmental DNA have reported that they correspond to a minor fraction of the total gene cassette pool. In fact, the majority of the gene cassettes found in those studies encode proteins with no homologous in the databases (Elsaied *et al.*, 2007; Koenig *et al.*, 2008; **Chapter 2**). These results strongly suggest that integrons may also have an important role in other cellular processes rather than just simply antibiotic resistance.

Similarly to slaughterhouse's effluent (**Chapter 3**), most integrases were located on bacterial chromosomes, although the prevalence of *intI* genes carried in

plasmids (33.3%) was higher in domestic wastewaters. Nevertheless, *intI1* genes were transferred into *E. coli* recipient cells at similar frequencies.

Urban effluents are known to contain high levels of antibiotics due to incomplete metabolism of excreted antibiotics (Baquero *et al.*, 2008; Xu *et al.*, 2007). Resistance to at least three antibiotics was observed in all isolates. Multiresistance to 5 and more antibiotics was displayed by 62.5% of *intI*-positive isolates. In both *Enterobacteriaceae* and *Aeromonas* spp., the most frequent resistances found were to ampicillin, cefalothin, erythromycin and nalidixic acid. In addition to species-specific penicillinases and cephalosporinases occurring in *Aeromonas* and *Enterobacteriaceae* species (Goñi-Urriza *et al.*, 2000), resistance to ampicillin and cefalothin may also be explained by the presence of extended spectrum β-lactamases that inactivate both penicillins and cephalosporins, such as bla_{GES}- and bla_{OXA}-type enzymes, detected in this study. On the other hand, macrolides, such as erythromycin, has been reported as one of the most frequently detected antibiotic at WWTPs (Xu *et al.*, 2007) and integrons embedded in erythromycin-resistance plasmids have been identified in municipal WWTP (Schlüter *et al.*, 2007a). Of particular concern is the high level of bacteria resistant to quinolones and fluoroquinolones observed in this study. Quinolones and fluoroquinolones, such as nalidixic acid and ciprofloxacin, are synthetic broad-spectrum antibiotics used in human and veterinary medicine and their persistence in the environment is due to their excretion as unchanged compounds (Cattoir *et al.*, 2008). Quinolone and fluoroquinolones resistance is usually due to DNA gyrase and topoisomerase IV mutations that render antibiotics ineffective (Cattoir *et al.*, 2008). Despite, emerging mechanisms of resistance such as *aacA4-CR* gene cassettes encoding fluoroquinolone acetylating aminoglycoside acetyltransferases have been identified in this study in plasmid-borne integrons in *A. media* and *A. allossacarophyla*, which might constitute a growing concern, since resistance to these compounds are rarely reported in *Aeromonas* spp. (Janda & Abbott, 2010).

In conclusion, results obtained suggest that the type of effluent affects both prevalence and diversity of integrons. In addition, 4 new gene cassette arrays were

found, including in the treated effluent, drawing attention to wastewaters, and in particular to domestic effluents, as sources and reservoirs of novel integron structures.

The high number of multiresistant isolates emphasizes the importance of wastewaters as vehicles of dissemination of antibiotic-resistant bacteria and the urgent need to employ effective means of effluent disinfection in wastewater treatment plants. Further investigation on the persistence of mobile genetic elements in wastewaters should continue in order to allow a sustainable use of resources, minimizing human impacts on aquatic ecosystems and the perpetuation and dissemination of harmful genetic determinants in the environment.

CHAPTER 5

Diversity of Gene Cassette Promoters in Class 1 Integrons from Wastewater Environments

5.1 Introduction

Integrons facilitate the acquisition and expression of new genetic determinants, being important players in bacterial adaptation (Guerin *et al.,* 2009).

The basic structure of an integron consists of an integrase gene (*intI*), responsible for the integration and excision of gene cassettes by site-specific recombination, a recombination site (*attI*) and one or two Pc promoters (Pc1-Pc2) responsible for the expression of gene cassettes located downstream (if present) (Cambray *et al.*, 2010).

It has been demonstrated that the regulation of integrase genes is dependent on the SOS response and that SOS induction controls the rates of cassette recombination (Guerin *et al.*, 2009). A LexA-binding motif ($CTGN_{10}CAG$) has been identified in integrons, overlapping the integrase putative promoter region (Guerin *et al.*, 2009). Under normal conditions, LexA represses *intI* transcription, maintaining gene cassette arrays in a steady state. The SOS response can be induced by several stresses such as the presence of antibiotics, leading to derepression of *intI* transcription and allowing the acquisition or reordering of gene cassettes by IntI integrase/excisase activity (Guerin *et al.*, 2009).

In class 1 integrons, Pc1 is located within the integrase gene and, as a consequence, any nucleotide substitutions at promoter level may affect IntI1 activity. Occasionally, class 1 integrons may also harbour a second promoter (Pc2), created by the insertion of a GGG triplet within the *attI1* site that optimizes the spacing between -35 and -10 boxes (from 14 nt to 17 nt), usually enhancing gene cassette expression (Jové *et al.*, 2010). In some cases, the presence of the second Pc2 compensates the weak strength of Pc1 without changing the activity of the

integrase (Jové *et al.*, 2010). Moreover, it has been hypothesized that the presence of Pc2, that overlaps LexA binding site, may result in the disruption of the later, leading to the possible constitutive expression of integrase (Jové *et al.*, 2010; Guerin *et al*, 2009).

Recently, Jové and co-workers (2010) have examined the capacity of different Pc1 variations and Pc1-Pc2 configurations to express a *lacZ* reporter gene. According to sequence homology with σ^{70} promoter consensus, four main Pc1 variants have been identified possessing different expression efficiencies: PcW (Weak, TGGACA-N_{17}-TAAGCT), PcH1 (Hybrid 1, TGGACA-N_{17}-TAAACT), PcH2 (Hybrid 2, TTGACA-N_{17}-TAAGCT) and PcS (Strong, TTGACA-N_{17}-TAAACT) (Jové *et al.*, 2010). In addition, mutations upstream the -10 hexamer, may also affect promoter efficiency. For instance, a TGN-10 motif upstream PcW increases its efficiency in about 15 fold, whereas TAN-10 motif significantly reduces its activity (Nesvera *et al.*, 1998; Jové *et al.*, 2010). However, evolutionary changes to stronger TGN-10 motifs may have great impact on integrase activity (Jové *et al.*, 2010). With few exceptions (PcS and PcW_{TGN-10}), the presence of the second promoter Pc2 (TTGTTA-N_{17}-TACAGT) also compensates weaker Pc1 variants, resulting in an increase in gene cassette expression without decreasing integrase activity (Collis & Hall, 1995; Jové *et al.*, 2010).

Altogether, thirteen Pc1 variants (four of which associated with a Pc2 promoter) have been identified based on -35 and -10 hexamers. According to their strength, the most frequent configurations are: PcW < PcW-Pc2 < PcH1 < PcH1-Pc2 < PcW_{TGN-10}-Pc2 < PcW_{TGN-10} < PcH2 < PcS-Pc2 < PS (Jové *et al.*, 2010).

It has also been shown that promoter strength inversely correlates with integrase activity: the weaker the promoter, the more active is the integrase excision activity (Collis & Hall, 1995; Jové *et al.*, 2010). Thus, information on promoter configuration may provide evidence not only for the level of expression of adjacent gene cassette arrays but also for the activity of integrase, which lead to gene cassette rearrangements.

Wastewaters have been reported as important reservoirs of integrons and gene cassettes (**Chapters 2-4**). In addition, it has been shown that the type of effluent affects both the prevalence and the diversity of gene cassettes arrays (**Chapter 2**; **Chapter 4**). In particular, slaughterhouse's effluents have been shown to possess an increased prevalence of integron-carrying bacteria comparing to domestic wastewaters (35% and 3.7%, respectively) (**Chapters 3-4**). Despite that, the diversity of gene cassette arrays present was found to be higher in bacteria isolated from domestic effluents (**Chapter 4**). As discussed before, these differences are thought to be due to the different selective pressures shaping the diversity of bacterial communities inhabiting those systems (**Chapter 2**; **Chapter 4**).

The aim of this study was to investigate the diversity of promoters involved in the expression of gene cassettes in class 1 integrons from wastewater environments.

5.2 Material and methods

The diversity of gene cassette promoters was investigated in 47 class 1 integrons detected in *Enterobacteriaceae* and *Aeromonas* spp. isolated from two distinct wastewater environments: urban wastewaters, consisting mostly of domestic effluents and a slaughterhouse wastewater, consisting of discharges with animal origin (**Chapters 3-4**).

Bacterial isolation, integron amplification and effluent characteristics have been previously described in detail (**Chapters 2-4**). Promoter sequences were identified manually according to Jové *et al.* (2010); additional (intra-array) putative promoters were searched using BPROM software (http://www.softberry.com/).

5.3 Results and discussion

Among 47 class 1 integrons, 6 different Pc1-Pc2 configurations were detected in at least one strain: PcS, PcH2, PcW_{TGN-10}, PcH1, PcW-Pc2 and PcW.

The distribution of the different Pc variants in class 1 integrons from the two wastewater systems is shown in **Figure 5.1**.

Results obtained showed the predominance of weaker Pc variants in both types of effluents. Nevertheless, the diversity of Pc1-Pc2 configurations differed according to type of wastewater: PcW largely prevailed in the slaughterhouse's collection whereas PcH1 was the most frequently detected variant in the urban wastewater (**Figure 5.1**). A higher diversity of Pc variants was observed in integrons from urban wastewater isolates: five different Pc1-Pc2 configurations were detected, namely PcH1 (43.5%), PcW (30.4%), PcW_{TGN-10} (13.0%), PcS (8.7%) and PcH2 (4.3%). In integrons from slaughterhouse's effluent, gene cassette promoters were limited to three configurations: PcW (79.2%), PcH1 (12.5%) and PcW-Pc2 (8.3%). Occurrence of the PcH1 variant in integrons from urban wastewaters as well as PcW variant in animal wastewater was much higher than those previously reported from *in silico* studies (26.2% and 16.5%, respectively; Jové *et al.*, 2010), though differences may be related to the exclusion of identical arrays from *in silico* data analysis.

It has been hypothesized that under intensive selective pressures, such as high concentrations of antibiotics and/or detergents, the need to express gene cassettes more efficiently could have led to the selection of stronger promoters (such as PcS an PcW_{TGN-10}) and to the stabilization of gene cassette arrays, due to lower excisase IntI1 activity (Jové *et al.*, 2010). Nevertheless, previous studies concerning clinical environments have also identified a high predominance of weak PcW variants in *Salmonella* (Lindstedt *et al.*, 2003; Schmitz *et al.*, 2001). Similarly, the predominance PcH1 was reported in *E. coli* isolates from human and animal origins (Cocchi *et al.*, 2007). In this study, the control of gene cassette expression in integrons from both wastewaters was associated to weaker Pc

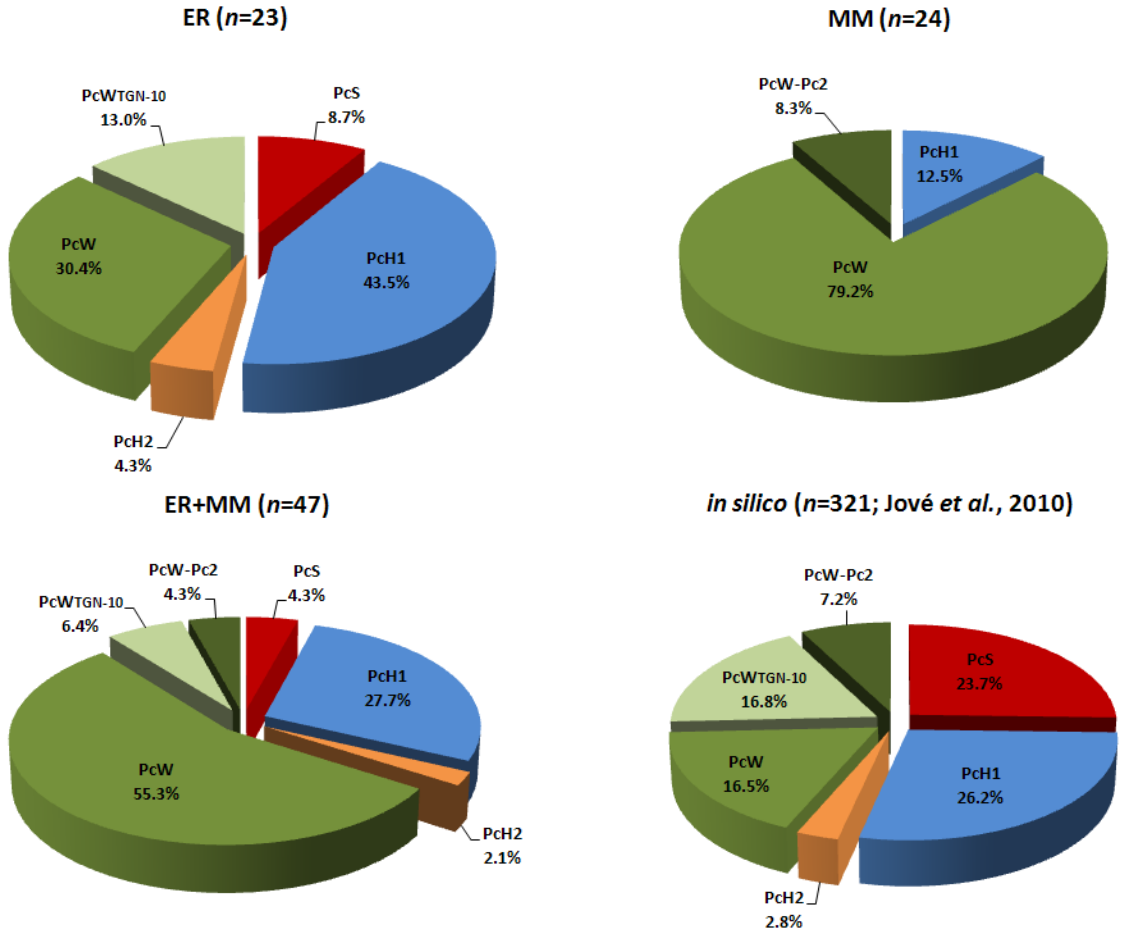


Figure 5.1 Occurrence of the different Pc variants in integrons from slaughterhouse's (MM) and urban (ER) wastewaters; previously reported occurrences based on GenBank *in silico* analyses (Jové *et al.*, 2010) are also represented.

variants (PcW and PcH1), suggesting the existence of a dynamic gene cassette pool in these environments. The presence of weaker promoters has been shown to be compensated with a higher capacity for gene cassette rearrangements, as opposed to stronger promoters that are associated with more stable gene cassette arrays (Jové *et al.*, 2010). This relates to the fact that variations at Pc1 level may generate different variants of the IntI1 enzyme, leading to similar integration efficiency, but quite different excision activities (Jové *et al.*, 2010).

As shown in **Table 5.1**, different promoters were found in integrons with identical gene cassette arrays, as in the case of integrons from strains *A. veronii* MM.1.10 and *A. caviae* ER.1.9, both carrying the *aadA2* gene cassette. Also, different Pc variants were not species-specific, as previously noted (Peters *et al.*, 2001). Nevertheless, some of wastewater strains exhibited promoter configurations never reported before in these genera. So far, identification of promoters in *Aeromonas* spp. had been limited to the PcW_{TGN-10}, PcH1 and PcW variants. Here, two additional (stronger) variants have been identified, PcS (*A. salmonicida* ER.1.7) and PcH2 (*A. media* ER.1.8). In *Enterobacteriaceae*, specifically in *Escherichia* and *Kluyvera* genera, additional configurations have also been found, namely PcW-Pc2 (*E. coli* MM.1.15 and *E. coli* MM.1.9) and PcW (*K. cryoscrescens* ER.1.27), respectively. These results strengthen previous observations that suggest that promoter diversity is independent of phylogeny and gene cassette arrays (Peters *et al.*, 2001). Nevertheless, more epidemiological studies focusing the analysis of promoter configurations will allow unravelling promoter ecology and constrains that may shape promoter diversity.

Table 5.1 Promoter configurations determined in this study.

Promoter configuration	Gene Cassette Array [1]	Strain		Resistance Phenotype
PcS	n.d.	*Klebsiella oxytoca*	ER.1.13	AMP, ERY, NAL, STR
	orfER.1.7::ISAs12 - aadA13	*Aeromonas salmonicida*	ER.1.7	AMP, CEF, NAL, STR
PcH2	*bla* $_{GES-1}$ *- aacA4*	*Aeromonas media*	ER.1.8	AMP, CEF, KAN, NAL, STR (CAZ, GEN)
PcW_{TGN-10}	*aacA4-CR - bla* $_{OXA-1}$ *- catB3 - arr3*	*Aeromonas allosaccharophila*	ER.1.4	AMP, CEF, ERY, GEN, KAN, NAL, STR (CIP)
	aacA4-CR - bla $_{OXA-1}$ *- catB3 - arr3*	*Aeromonas media*	ER.1.25	AMP, CEF, CIP, ERY, NAL
	dfrA12 - orfF - aadA2	*Aeromonas media*	ER.1.1	AMP, CEF, ERY, KAN, NAL, STR, STX
PcH1	*aacA4-CR - bla* $_{OXA-1}$ *- catB3 - arr3*	*Aeromonas allosaccharophila*	ER.1.16	AMP, CEF, ERY, GEN, KAN, NAL, STR, TET
	n.d.	*Enterobacter cloacae*	ER.1.10	AMP, CEF, ERY (STR)
	aacA4 - catB8 - aadA1	*Aeromonas allosaccharophila*	ER.1.16	AMP, CEF, ERY, GEN, KAN, NAL, STR, TET
	bla $_{OXA-2}$ *- aadA1 - bla* $_{OXA-2}$ *- orfD*	*Aeromonas caviae*	ER.1.26	AMP, CEF, NAL, STR (ERY)
	aadA1	*Aeromonas jandaei*	MM.1.24	AMP, CEF, ERY, IPM, STR
	aadA1	*Aeromonas media*	ER.1.5	AMP, CEF, ERY, KAN, NAL, STR, STX (CHL)
	aadA2	*Aeromonas media*	ER.1.11	AMP, CEF, NAL, STR (ATM, STX, ERY)
	aacA4 - catB3 - bla $_{OXA-10}$ *- aadA1*	*Aeromonas media*	ER.1.17	AMP, CEF, KAN, NAL, STR, TET (STX, ERY)
	catB8 – aadA17	*Aeromonas media*	ER.1.18	AMP, CAZ, CEF, NAL, STR
	catB3 - aadA1	*Aeromonas* sp.	MM.1.6	AMP, ERY, IPM, STR, TET (CEF, STX)
	aadA1	*Aeromonas veronii*	ER.1.24	AMP, CEF, ERY, KAN, NAL, STR
	aadA2	*Aeromonas veronii*	MM.1.10	AMP (ERY, STR, TET)
	dfrA17 – aadA5	*Shigella* sp.	ER.1.23	AMP, CEF, CIP, ERY, NAL, STR, STX, TET
PcW-Pc2	*aadA1*	*Escherichia coli*	MM.1.15	ERY, STR, TET
	aadA1	*Escherichia coli*	MM.1.9	CEF, ERY, STR, TET
PcW	*dfrA1 - aadA1*	*Aeromonas salmonicida*	MM.1.19	AMP, CEF, STR, STX, TET (ERY)
	dfrA1 - aadA1	*Aeromonas salmonicida*	MM.1.3	AMP, CEF, STR, STX (ERY)
	dfrA1 - aadA1	*Aeromonas salmonicida*	MM.1.4	AMP, CEF, STR, STX, TET (ERY)
	dfrA1 - aadA1	*Aeromonas salmonicida*	MM.1.23	AMP, CEF, STR, STX, TET (ERY)
	dfrA1 - aadA1	*Aeromonas salmonicida*	MM.1.29	AMP, CAZ, CHL, CEF, ERY, GEN, IPM, STR, STX, TET
	n.d.	*Aeromonas* sp.	ER.1.21	AMP, CAZ, CEF, ERY, NAL
	dfrA1 - aadA1	*Aeromonas* sp.	MM.1.8	AMP, CEF, STR, STX, TET (ERY)
	dfrA1 - aadA1	*Escherichia coli*	MM.1.5	AMP, CEF, ERY, STR, STX, TET
	aadA2	*Aeromonas caviae*	ER.1.9	ATM, CAZ, NAL (STR)
	bla $_{OXA-1}$ *- aadA1*	*Escherichia coli*	MM.1.12	AMP, ERY, STR, TET (CEF)
	dfrA1 - aadA1	*Escherichia coli*	MM.1.11	ERY, TET, STX (CEF, STR)
	dfrA1 - aadA1	*Escherichia coli*	MM.1.13	AMP, CEF, CHL, ERY, STR, STX, TET
	dfrA1 - aadA1	*Aeromonas allosaccharophila*	ER.1.6	AMP, CEF, NAL, STR, STX (IPM, ERY)
	empty integron	*Aeromonas caviae*	ER.1.2	AMP, CAZ, CEF, ERY, KAN, NAL, STR (CHL, CIP)
	dcyA	*Aeromonas caviae*	ER.1.20	AMP, CEF, NAL
	empty integron	*Aeromonas media*	ER.1.22	AMP, CEF, CHL, CIP, ERY, NAL, TET (STR, KAN)
	dfrA1 - aadA1	*Aeromonas salmonicida*	MM.1.2	AMP, CEF, STR, STX, TET (ERY)
	dfrA1 - aadA1	*Aeromonas salmonicida*	MM.1.16	AMP, CEF, STR, STX, TET (ERY)
	dfrA1 - aadA1	*Aeromonas salmonicida*	MM.1.17	AMP, CEF, STR, STX, TET (ERY)
	dfrA1 - aadA1	*Aeromonas salmonicida*	MM.1.18	AMP, CEF, STR, STX (ERY, TET)
	dfrA1 - aadA1	*Aeromonas salmonicida*	MM.1.20	AMP, CEF, STR, STX, TET (ERY)
	dfrA1 - aadA1	*Aeromonas salmonicida*	MM.1.22	AMP, CEF, STR, STX, TET (ERY)
	dfrA1 - aadA1	*Aeromonas salmonicida*	MM.1.26	AMP, CEF, STR, STX, TET (ERY)
	aadA2	*Aeromonas* sp.	MM.1.1a	AMP, CEF, CHL, STR, TET (ERY)
	n.d.	*Aeromonas veronii*	MM.1.27	AMP (ERY)
	empty integron	*Kluyvera cryocrescens*	ER.1.27	AMP, CEF, CIP, ERY, NAL

[1] n.d.: not detected

In conclusion, results obtained showed the predominance of the weak promoter variants in both types of effluents. However, the PcW variant largely prevailed in integrons from animal's wastewaters, while PcH1 was prevalent in domestic effluents. Interestingly, these weaker promoters are known to determine the most efficient integron integrases for recombination of gene cassettes. Moreover, the prevalence of the Pc variants detected in this study was higher than those previously reported, mostly concerning integrons from clinical origin. Nevertheless, we are aware that the number of integrons analysed in this study is limited and that different methodologies applied among published studies may vary.

To the best of our knowledge this constituted the first investigation concerning the configuration of gene cassette promoters in integrons from wastewater environments. Results obtained provide insights into the gene cassette dynamics in integrons from wastewater environments. Other environments should be investigated in order to clarify whether the Pc diversity correlate with specific environmental conditions.

CHAPTER 6

R-Plasmid Diversity among *Enterobacteriaceae* and *Aeromonas* spp. Isolated from Wastewater Environments

6.1 Introduction

Identification and classification of plasmids has been an important issue in the last decades to trace plasmid evolutionary origins and to elucidate their role in environmental processes and microbial adaptation (Johnson & Nolan, 2009). Classification is usually based on genetic traits related with plasmid maintenance and replication control. Replicon typing classifies plasmids by their ability to stably coexist with other plasmids in the same bacterial strain. Plasmids that use the same replication system belong to the same incompatibility group and compete for stable maintenance. Therefore plasmids belonging to the same incompatibility group cannot coexist in the same cell, although their accessory genes may be different (Couturier *et al.*, 1988).

The importance of plasmids in bacteria adaptation has been reported in several environments, such as soil (Lee *et al.*, 2006), river and marine waters (Shintani *et al.*, 2008; Thavasi *et al.*, 2007), phytosphere (Tett *et al.*, 2007) and wastewaters (Verma *et al.*, 2002). Despite the energetic burden, plasmids provide fitness advantage to their hosts which allow them to persist across bacterial generations (Dionísio *et al.*, 2005). The genetic traits harboured on plasmids may include genes involved in mechanisms such as resistance, energy metabolism, virulence, pathogenicity, symbiosis and/or dissemination, favouring the survival of bacterial hosts under selective pressures (Dionísio *et al.*, 2002; Kado, 1998).

Conjugation is considered a major pathway for horizontal gene transfer among bacteria (Sørensen *et al.*, 2005). It involves direct cell-to-cell contact and DNA exchange usually mediated by a conjugative plasmid. Conjugative plasmids are highly promiscuous and transfer may occur between genera or even different

kingdoms (Ochman *et al.*, 2000). Resistance plasmids (R-plasmids) are found in several bacterial genera, both Gram-negative and Gram-positive. Several plasmid families carrying multiple antibiotic resistance determinants have been reported in *Aeromonas* spp. (Sørum *et al.*, 2003; Picão *et al.*, 2008; Fricke *et al.*, 2009) and *Enterobacteriaceae* isolates (Carattoli, 2009; Carattoli *et al.*, 2010). Because of their wide distribution and because they confer multiple antibiotic resistance, R-plasmids are of both clinical and environmental concern.

Wastewater treatment plants (WWTPs) are important reservoirs of resistance determinants and favourable places for horizontal gene transfer (HGT), due to high microbial abundance, high nutrient concentrations and intense selective pressures imposed by antibiotics, detergents and other pollutants (**Chapters 3-4**; Lindberg *et al.*, 2007; Schwartz *et al.*, 2003). Moreover, it has been proposed that sub-inhibitory antibiotic concentrations may also act as cell signalling agents, inducing biofilm formation, as observed in the opportunistic pathogen *Pseudomonas aeruginosa* when exposed to sub-inhibitory concentrations of tobramycin, tetracycline and ciprofloxacin (Linares *et al.*, 2006). Biofilm formation increases the rate of gene transfer and the expression of conjugative proteins helps to establish high-density bacterial biofilms (Ghigo, 2001). As a result, WWTPs may act as sources of dissemination of undesirable genetic traits, such as those coding for antibiotic resistant determinants, to natural waters, soils and eventually the food chain.

In the previous chapters, the presence and distribution of integron-carrying bacteria was investigated at the different stages of the treatment process in two wastewater treatment plants, one treating urban discharges and other treating wastewater from a slaughterhouse (**Chapters 3-4**). Even though integrons are transposition defective, they are often mobilizable in association with functional transposons and/or conjugative plasmids, as revised in **Chapter 1**.

The present study was performed to better address the diversity of resistance (R) - plasmids in *intI*-positive strains retrieved from wastewaters, in

order to gather further data pertaining to the contribution of these environments to HGT and spread of integrons and antibiotic resistance determinants.

6.2 Material and methods

6.2.1 Bacterial strains and mating assays

Sixty-six strains belonging to *Aeromonas* sp. (*n*=48) and *Enterobacteriaceae* (*n*=18) collected from urban and slaughterhouse wastewaters (**Chapters 3-4**) were included as donors in mating assays using rifampicin- and kanamycin resistant *Escherichia coli* CV601-GFP and *Pseudomonas putida* KT2442-GFP as recipient strains (Smalla *et al.*, 2006).

Liquid cultures of donor and recipient strains were prepared separately in 10 mL of Luria-Bertani broth (LB) and grown overnight with gentle shaking at 28°C. Recipient and donor strains were mixed (ratio 1:1) and centrifuge 5 min at 10000 rpm to precipitate cells. Supernatant was discarded and replaced by 1 mL of fresh LB. Mixtures were incubated overnight at 28°C without shaking. Cells were then precipitated by centrifugation (5 min, 10000 rpm) and washed in 0.9% NaCl solution. Serial dilutions were prepared in 0.9% NaCl and aliquots of 100 µL were spread on Plate Count Agar (PCA) plates supplemented with rifampicin (50 $mg.L^{-1}$ in methanol) + streptomycin (50 $mg.L^{-1}$) or rifampicin (50 $mg.L^{-1}$ in methanol) + tetracycline (50 $mg.L^{-1}$). Putative transconjugants were grown at 28°C for 48h. Assays were run in duplicate. Donor and recipient were also placed on the selective plates for mutant detection.

Putative transconjugants were confirmed by BOX-PCR typing. Profiles were generated by PCR amplification in 25 µL reaction mixtures containing 3.75 mM MgCl2, 0.2 mM dNTPs, 1x Stoffell Buffer, 0.2 µM of primer BOX-AIR (5'-CTA CGG CAA GGC GAC GCT GAC G-3'; Versalovic *et al.*, 1994), 2.5 U Stoffel *Taq*

polymerase (Applied Biosystems, USA) and 1 µL of cell suspension prepared in 100 µL of distilled water (~1.0 McFarland turbidity standard).

Amplification was carried out as follows: initial denaturation for 7 min at 94ºC, then 35 cycles of denaturation at 94ºC for 7 min , followed by annealing at 53ºC for 1 min and extension at 65ºC for 8 min, and a final extension at 65ºC for 16 min.

6.2.2 Plasmid isolation and restriction analysis

Plasmid DNA from donors and transconjugants was purified using QIAGEN Plasmid Mini-Kit (QIAGEN GmbH, Germany). 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 manufacturer's instructions (Fermentas, Lithuany).

Restriction patterns were visualized in 0.8% agarose gels. Electrophoresis was run at 40 V for 3 h in 0.5x TBE buffer (50 mM Tris, 50 mM boric acid, 0.5 mM ethylenediaminetetraacetic acid) and stained using ethidium bromide. Restriction patterns were compared using of GelCompar II software (Applied Maths, SintMartens-Latem, Belgium).

6.2.3 Southern-blot and hybridization with replicon probes

Gels were transferred onto nylon membranes (Hybond-N, Amershan, Germany) and hybridized in middle stringency conditions with replicon-specific digoxigenin-labbeled probes for IncP-1 (*trfA*), IncQ (*oriV*), IncN (*rep*) and IncW (*oriV*) in order to determine their incompatibility group, as previously described (**Chapter 2**; Bihn *et al.*, 2008).

6.3 Results and discussion

In order to gain insight into the presence and diversity of other mobile genetic elements in integron-carrying bacteria from wastewater environments, the presence and diversity of plasmids was assessed in this study. The presence of pDNA was confirmed through plasmid purification and gel electrophoresis in 68% (45 out of 66) donor strains. In the remaining 12 strains (~32%) no plasmids were present or were not retrieved using the described methodology. Thus, the majority of strains analysed harboured at least one plasmid and the presence of pDNA was detected in strains retrieved from all stages of the treatment process (**Table 6.1**), indicating that the treatment system does not effectively eradicate R-plasmids from final effluents. As suggested previously, the high availability of nutrients in wastewater environments may favour biofilm formation (Sørensen *et al.*, 2005), increasing the chances of cell-to-cell contact and encouraging the occurrence of HGT throughout the treatment process.

Plasmid restriction analysis showed a wide and diverse plasmid pool present in these strains and in total 45 different restriction patterns (similarity < 98%) were obtained (**Figure 6.1)**. Restriction patterns did not cluster by species, type of effluent or treatment stage, suggesting a high diversity of backbones and/or accessory elements. Results obtained thus reinforce the role of wastewaters as reservoirs of diverse mobile genetic elements and hot spots for HGT, as previously discussed (**Chapters 2-4**; Schlüter *et al.*, 2007; Baquero *et al.*, 2008).

Table 6.1 Characterization of donor strains used in this study in terms of phylogeny, presence at wastewater treatment plants and generation of transconjugants in mating assays.

Donor Strains[a]		Presence at WWTP[b]	Generation of Transconjugants[c]
Aeromonadaceae (n=48)			
Aeromonas allosaccharophila	ER.1.4	RW	Ec/STR
Aeromonas allosaccharophila	ER.1.16	FE	Pp/STR
Aeromonas allosaccharophila	MM.1.1	FE	Ec/STR
Aeromonas caviae	ER.1.26	PD	Ec/STR; Ec/TET
Aeromonas caviae	MM.1.25	FE	Ec/STR
Aeromonas jandaei	MM.1.24	SR	Ec/STR; Pp/STR
Aeromonas media	ER.1.1	RW	Pp/STR
Aeromonas media	ER.1.5	RW	Ec/STR
Aeromonas salmonicida	MM.1.2	AT, SR, FE	Pp/TET
Aeromonas salmonicida	MM.1.3	SR, FE	Ec/STR; Pp/STR; Pp/STR
Aeromonas salmonicida	MM.1.4	AT, SR, FE	Pp/TET
Aeromonas salmonicida	MM.1.16	AT	Pp/TET
Aeromonas salmonicida	MM.1.17	AT	Pp/TET
Aeromonas salmonicida	MM.1.18	AT	Ec/STR; Pp/STR
Aeromonas salmonicida	MM.1.19	AT	Ec/STR
Aeromonas salmonicida	MM.1.20	AT	Pp/TET
Aeromonas salmonicida	MM.1.22	SR	Ec/STR; Pp/TET
Aeromonas salmonicida	MM.1.23	SR	Pp/STR; Pp/TET
Aeromonas salmonicida	**MM.1.26**	FE	Ec/STR
Aeromonas salmonicida	MM.1.28	FE	Pp/STR
Aeromonas salmonicida	MM.1.29	FE	Pp/TET
Aeromonas sp.	MM.1.1a	HT	Pp/TET
Aeromonas sp.	MM.1.6	SR	Pp/TET
***Aeromonas* sp.**	**MM.1.8**	AT	Ec/STR
Aeromonas sp.	MM.2.0	HT	Pp/TET
Aeromonas sp.	MM.2.5	HT	Pp/STR
Aeromonas sp.	MM.2.10	FE	Pp/STR
Aeromonas veronii	ER.1.24	PD	Ec/STR
Aeromonas allosaccharophila	ER.1.6	RW	-
Aeromonas caviae	ER.1.2	RW	-
Aeromonas caviae	ER.1.9	AT	-
Aeromonas caviae	ER.1.20	FE	-
Aeromonas hydrophila	MM.1.21	SR	-
Aeromonas media	ER.1.8	RW	-
Aeromonas media	ER.1.11	AT	-
Aeromonas media	ER.1.17	FE	-
Aeromonas media	ER.1.18	FE	-
Aeromonas media	ER.1.19	FE	-
Aeromonas media	ER.1.22	PD	-
Aeromonas media	ER.1.25	PD	-
Aeromonas media	MM.2.4	HT	-
Aeromonas media	MM.2.9	AT	-
Aeromonas salmonicida	ER.1.7	RW	-
Aeromonas sp.	ER.1.21	PD	-
Aeromonas sp.	MM.2.7	HT	-
Aeromonas veronii	MM.1.10	HT	-
Aeromonas veronii	MM.1.27	FE	-
Aeromonas veronii	MM.2.8	AT	-

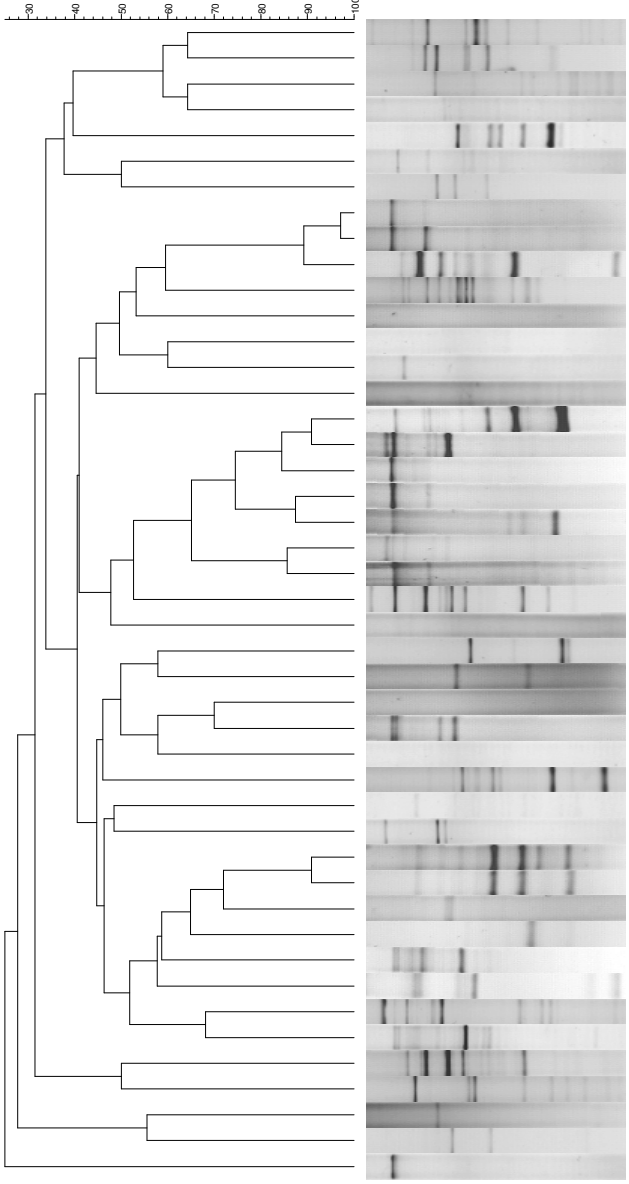
Table 6.1*Continued*

Donor Strains[a]		Presence at WWTP[b]	Generation of Transconjugants[c]
Enterobacteriaceae (n=18)			
Escherichia coli	**MM.2.2**	RW	Ec/TET
Escherichia coli	**MM.1.12**	RW	Ec/STR; Ec/TET
Escherichia coli	**MM.1.9**	HT	Ec/STR; Ec/TET
Escherichia coli	**MM.1.7**	SR	Ec/STR; Pp/STR; Pp/TET
Escherichia coli	**MM.1.5**	FE	Ec/STR
Escherichia coli	MM.2.6	HT	Ec/STR
Escherichia coli	MM.2.11	FE	Ec/STR; Pp/TET
Escherichia coli	MM.1.11	RW	Pp/TET
Escherichia coli	**MM.1.13**	RW	Ec/STR
Escherichia coli	**MM.1.14**	RW	Ec/TET
Escherichia coli	**MM.1.15**	RW	Ec/TET
Morganella morganii	MM.2.3	RW	Ec/STR; Pp/TET
Shigella sp.	ER.1.23	PD	Pp/STR; Pp/TET
Enterobacter cloacae	ER.1.10	AT	-
Enterobacter sp.	ER.2.3	AT	-
Escherichia coli	MM.2.1	RW	-
Klebsiella oxytoca	ER.1.13	AT	-
Kluyvera cryocrescens	ER.1.27	FE	-

[a] Strains MM# were obtained from slaughterhouse's wastewaters, whereas strains ER# refer to urban wastewaters; **strains that generated *intI*$^+$-transconjugants are highlighted in bold.**

[b] Presence at wastewater treatment plant: RW, raw waters; HT, homogenization tank; PD, primary decantation; SR, sludge recirculation; FE, final effluent;

[c] Ec (*E. coli* CV601-GFP) and Pp (*P. putida* KT2422-GFP) refer to the recipient strains; TET (tetracycline) and STR (streptomycin) refer to the selective markers used; -, no transconjugants observed;



Donor Strain		Dispersion at WWTP	Incompatibility Group
A.media	ER.1.17	FE	n.d.
A.media	ER.1.19	FE	n.d.
A.media	ER.1.11	AT	n.d.
A.media	ER.1.18	FE	n.d.
Enterobacter sp.	ER.2.3	AT	n.d.
Aeromonas sp.	ER.1.21	PD	n.d.
A.media	ER.1.25	PD	n.d.
A.salmonicida	MM.1.2	AT, SR, FE	n.d.
A.salmonicida	MM.1.4	AT, SR, FE	n.d.
A.salmonicida	MM.1.3	SR, FE	n.d.
A.caviae	ER.1.9	AT	n.d.
Aeromonas sp.	MM.1.6	SR	n.d.
K.oxytoca	ER.1.13	AT	n.d.
A.media	ER.1.22	PD	n.d.
A.veronii	ER.1.24	PD	n.d.
A.salmonicida	MM.1.17	AT	n.d.
A.salmonicida	MM.1.19	AT	n.d.
A.salmonicida	MM.1.23	AT	n.d.
A.salmonicida	MM.1.20	AT	n.d.
A.salmonicida	MM.1.22	AT	n.d.
A.salmonicida	MM.1.16	AT	n.d.
A.salmonicida	MM.1.18	AT	n.d.
K.cryocrescens	ER.1.27	FE	n.d.
A.allosacharophila	ER.1.16	FE	n.d.
A.allosacharophila	ER.1.6	RW	n.d.
A.allosacharophila	MM.1.1	FE	n.d.
Aeromonas sp.	MM.1.1a	HT	n.d.
A.jandaei	MM.1.24	AT	n.d.
E.cloacae	ER.1.10	AT	n.d.
A.allosacharophila	ER.1.4	RW	n.d.
Shigella sp.	ER.1.23	PD	n.d.
A.media	ER.1.5	RW	n.d.
E.coli	MM.1.5	FE	n.d.
Aeromonas sp.	MM.1.8	AT	n.d.
E.coli	MM.1.9	HT	n.d.
E.coli	MM.1.7	SR	n.d.
E.coli	MM.2.1	RW	n.d.
E.coli	MM.2.2	RW	n.d.
A.media	ER.1.8	RW	n.d.
E.coli	MM.2.11	FE	n.d.
A. media	ER.1.1	RW	n.d.
A.salmonicida	ER.1.7	RW	n.d.
A.caviae	ER.1.2	RW	n.d.
A.caviae	ER.1.26	PD	n.d.
A.caviae	ER.1.20	FE	n.d.

Figure 6.1 UPGMA dendrogram based on Dice similarity correlation generated from the plasmid restriction patterns of donor strains obtained from urban (*n*=24) and slaughterhouse (*n*=21) wastewaters. Incompatibility grouping was determined by hybridization with IncN, IncW, IncQ and IncP-1 probes (n.d., not detected). Dispersion at wastewater treatment plant (WWTP) is also indicated: RW, raw waters; PD, primary decantation tank; HT, homogenization tank; AT, aeration tank; SR, sludge recirculation; FE, final effluent.

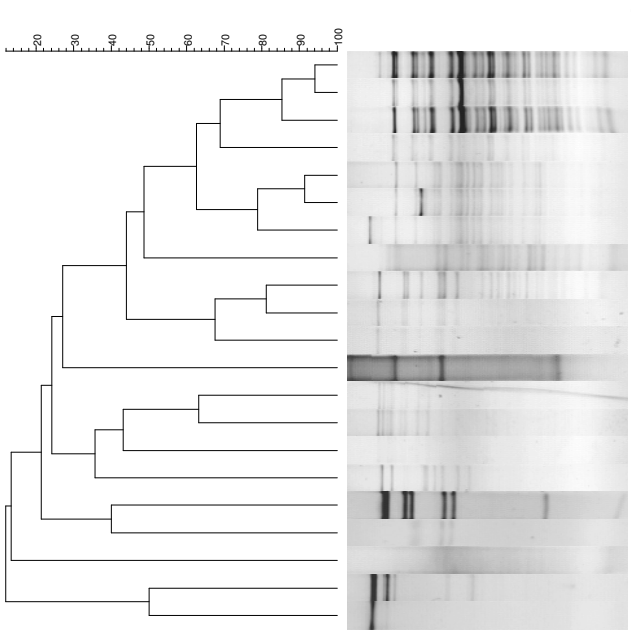
To investigate the conjugative nature of such plasmids, all strains were further used as putative donors in mating assays. Among *Aeromonas* sp. isolates, ~58% (28 out of 48) donor strains transferred successfully to recipient strains. Among *Enterobacteriaceae* isolates, up to 72% (13 out of 18) donors produced transconjugants. In total, approximately 62.1% (41 out of 66) of donor strains transferred successfully to recipient strains, whereas 37.9% (25 out of 66) did not produce any transconjugants with neither of the recipient strains and conditions tested. The ability of plasmids to pass between distantly related species has been reported as of lower efficiency comparing with their ability to transfer between similar bacteria, due to diverse impediments such as restriction systems (Schafer *et al.* 1994; Sanderson, 1996). Despite, both *Aeromonas* and *Enterobacteriaceae* strains have generated transconjugants using *E. coli* and *P. putida* as recipient strains (**Table 6.1**).

The generation of transconjugants was observed in strains from all stages of the treatment process, including final effluent (**Table 6.1**). None of transconjugants gave positive hybridization signals using probes targeting IncN, IncQ, IncW or IncP-1 broad-host-range plasmids (**Figure 6.1**), though the presence of such groups had been detected in total community DNA (**Chapter 2**). Other studies dealing with total community DNA and exogenous isolation of plasmids from urban wastewaters also suggested that plasmids, in particular those belonging to IncP-1 group, are abundant in wastewater environments (Dröge *et al.*, 2000; Schülter *et al.*, 2007; Bahl *et al.*, 2009a).Thus, these discrepancies may suggest that (i) the diversity of plasmids retrieved in this study may possess a narrow-host range and/or (ii) the presence of broader host-range plasmids may probably be attributed to bacteria from other classes than those focused in this study. Further experiments using probes targeting other plasmid incompatibility groups, such as IncU and IncA/C groups that have been frequently reported in *Aeromonas* and *E. coli* isolates, will allow clarifying this situation.

Contrarily to the results obtained in **Chapters 3-4,** no transconjugants were obtained for strains MM.1.3, MM.2.11 and MM.2.6. This may be due to the use of

different methodologies, such as temperature of incubation and additional centrifugation steps, that may affect pili formation or integrity and plasmid stability (Friehs, 2004; Bahl *et al.*, 2009b). As discussed before, the establishment of a standardize methodology for plasmid transfer analysis would be recommended in order to allow the systematically testing of conjugative transfers in microbial populations (Sørensen *et al.*, 2005).

Accessory genetic modules, such as integrons, are integrated among functional plasmid backbone modules. Overall, 15% (10 out of 66) of donors analysed using this methodology harboured plasmid-borne integrons. Results were consistent to those obtained in **Chapters 3-4.** Similarly, Tennstedt and co-workers (2003) reported the presence of ~12% of class 1 integrons in 97 resistant plasmids obtained by exogenous isolation from an urban wastewater treatment plant.



Conjugative Plasmid	Incompatibility Group	Selective Marker	Donor Strain	
pEc10	n.d.	STR	*Aeromonas* sp.	MM.1.8
pEc9	n.d.	STR	*Aeromonas* sp.	MM.1.8
pEc5	n.d.	STR	*E. coli*	MM.1.5
pEc11	n.d.	STR	*Aeromonas* sp.	MM.1.8
pEc13	n.d.	STR	*E. coli*	MM.1.9
pEc17	n.d.	TET	*E. coli*	MM.1.9
pEc14	n.d.	STR	*E. coli*	MM.1.9
pEc31	n.d.	TET	*E. coli*	MM.1.15
pEc20	n.d.	STR	*E. coli*	MM.1.12
pEc41	n.d.	STR	*A. salmonicida*	MM.1.26
pPp18	n.d.	STR	*E. coli*	MM.1.7
pEc44	n.d.	TET	*E. coli*	MM.2.2
pEc24	n.d.	STR	*E. coli*	MM.1.13
pEc25	n.d.	STR	*E. coli*	MM.1.13
pEc28	n.d.	TET	*E. coli*	MM.1.14
pEc23	n.d.	TET	*E. coli*	MM.1.12
R751	**IncP1β**			
RF1010	**IncQ**			
pEc30	n.d.	TET	*E. coli*	MM.1.15
RN3	**IncN**			
IR388	**IncW**			

Figure 6.2 UPGMA dendrogram based on Dice similarity correlation generated from the plasmid restriction patterns of *intI*$^{+}$-conjugative plasmids obtained in this study. Incompatibility grouping was determined by hybridization with IncN, IncW, IncQ and IncP-1 probes (n.d., not detected); reference plasmids are highlighted in bold. TET (tetracycline) and STR (streptomycin) refer to the selective markers used in mating assays.

The diversity of restriction patterns obtained from *intI*-positive conjugative plasmids is shown in **Figure 6.2**.

The majority of patterns obtained showed low similarity (< 25%) with the reference IncP-1, IncQ, IncN and IncW plasmids used in this study, suggesting once again that other incompatibility groups should be investigated in order to characterize the nature of these replicons.

The presence of plasmid-borne integrons facilitates their dissemination through bacterial conjugation. Moreover, it has been recently demonstrated that conjugative plasmid transfer may also induce the expression of integron integrase, due to the formation of *ssDNA* during plasmid transfer that generally induces the bacterial SOS response (Baharoglu *et al.*, 2010). As a consequence, conjugative plasmid transfer may trigger gene cassette recombination (Baharoglu *et al.*, 2010). Continued study on these plasmids should provide additional insights into their genetics, evolution, diversity and role in integron dissemination. Future work focusing on comprehensive plasmid typing and sequencing of selected plasmids will allow elucidating the diversity of backbones and accessory modules occurring in these environments. Additionally, information on gene sequence may help to clarify the role of these structures in wastewater treatment process.

CHAPTER 7

Bioinformatics (2009) 25: 1096–1098

INTEGRALL: A Database and Search Engine for Integrons, Integrases and Gene Cassettes

7.1. Introduction

Prokaryotic genomes are characterised by a high degree of plasticity and an enormous evolutive potential (van Passel *et al.*, 2008). One of the major forces contributing to genome evolution is the exogenous acquisition of genetic material, so-called horizontal gene transfer (HGT). HGT contributes to adaptation to different ecological niches in response to selective pressures generating novel gene combinations (Sørensen *et al.*, 2005). HGT may overcome species boundaries and has been implicated in the dispersion of genes involved in complex cellular processes, including pathogenicity (Hentschel & Hacker, 2001).

The high number of studies regarding mobile genetic elements (MGE) has quickly generated a huge amount of information concerning these structures. As a consequence of this informative burst, two bottlenecks are limiting the easy access and analysis of molecular sequences representing mobile elements: confusing nomenclatures and absence of filtration of public databases to eliminate non reliable data. To overcome that, databases dedicated to MGE such as plasmids and phages (ACLAME; Leplae *et al.*, 2004) and insertion sequences (ISFinder; Siguier *et al.*, 2006) have been created.

Two decades have passed since the discovery of integrons but despite their importance and their extraordinary diversity of organization there is no database dedicated to the deposition and annotation of molecular data concerning these structures. Integrons are mobilisable platforms that play a key role in the enhancement of genetic diversity in bacteria. They act as bacterial recombination systems that mediate the capture and expression of gene cassettes (Hall *et al.*, 1999). Integrons are considered as the primary mechanism for antibiotic resistance

gene acquisition among bacteria and are frequently associated with transposons and conjugative plasmids (Gillings *et al.*, 2008). Integrons are composed of an integrase encoding gene (*intI*), a recombination site (*attI*) and one or two promoters that control the expression of gene cassettes (Hall *et al.*, 1999). Their genetic organization can be quite diverse, with different combinations of gene cassettes. In bacterial communities integrons also represent an important fraction of the mobile gene pool (Mazel, 2006). Furthermore, integrons are present in about 9% of completely or partially sequenced genomes (Boucher *et al.*, 2007). For a long time and until recently, research on integron diversity had been restricted to bacterial isolates obtained mainly from clinical sources. However, recent studies report the existence of natural environments as reservoirs of gene cassettes and sources of functionally diverse gene products often recovered by methods that access the non-cultivable bacterial fraction (**Chapter 2-4**; Boucher *et al.*, 2007)

To overcome one of the difficulties produced by the disclosure of many new sequences, handling and managing data, a database dedicated to integrons was projected. The INTEGRALL web-based platform was developed by microbiologists in collaboration with computer scientists. INTEGRALL aims to be a repository for 20 years of data about integrons, gathering information on the phylogeny of the bacterial hosts and their ecology, the molecular diversity of inserted gene cassettes and the type of integrases. The correlation between this data is essential to understand the role of integrons in bacterial adaptive responses and interactions.

7.2 The site

INTEGRALL was developed in PHP 5, using a MySQL 5.0 database backend. Implementation uses standard PC servers, with a Linux based distribution and Apache 1.3 server running PHP as FastCGI. **Figure 7.1** shows INTEGRALL web-interface already available at http://integron.bio.ua.pt.

The current version of INTEGRALL (1.2) includes:

i. Background information regarding integrons;
ii. *LIST* tool;
iii. A *SEARCH* tool and BLAST interface;
iv. Guidelines for nomenclature of gene cassettes;
v. A *GLOSSARY* of integron-associated genes;
vi. A *FORUM* discussion on mobile genetic elements.

The *LIST* tool provides a table of all integrase genes and/or gene cassette arrays deposited at NCBI GenBank (http://www.ncbi.nlm.nih.gov/sites/entrez), excluding redundant entries.

For every entry, information regarding identification of the bacterial host, type of integrase and gene cassettes arrays (if present) is provided. Other remarks may also be included in this section, such as integron type and associated mobile genetic elements. Additional data and functions like information about the source of isolation or direct access to FASTA sequences, together with bibliographic references are also available. When possible, users also have the possibility to access information concerning the genetic context of integrons (as in the case of completely sequenced plasmids and genomes) by pushing the *context* button. Moreover, all entries have a direct link to the complete gene sequence and additional information deposited at NCBI Genbank.

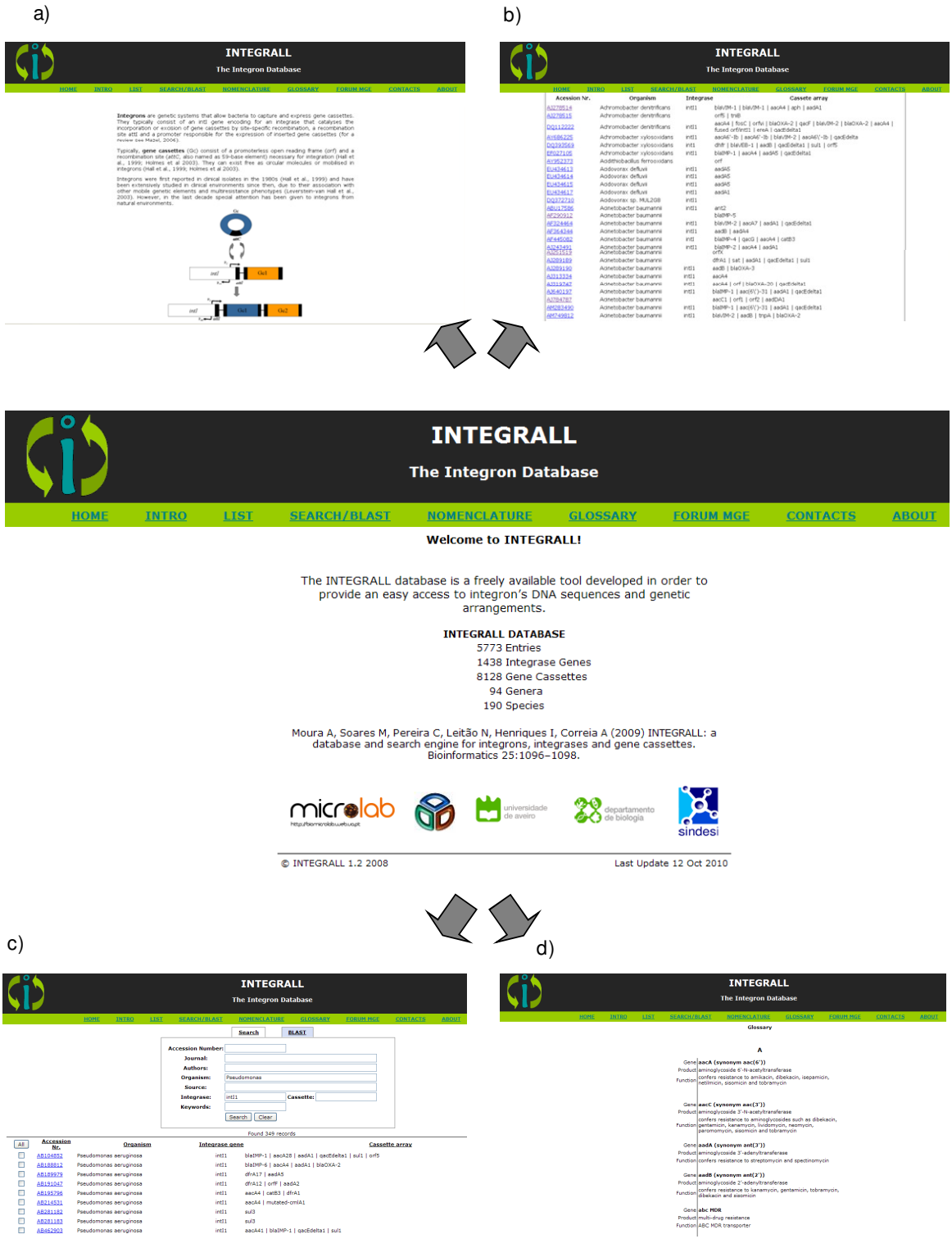


Figure 7.1 INTEGRALL web-interface available at http://integron.bio.ua.pt: a) INTRO section, b) LIST section, c) SEARCH section, d) GLOSSARY section.

The *SEARCH* tool allows two modes of operation: simple or advanced search. Used in advanced mode, *SEARCH* is able to simultaneously combine two or more of the following criteria: accession number, journal, authors, organism and source of isolation, integrase gene or gene cassettes. Other features, such as other integron associated genes and integron genomic location (plasmid and/or chromosome) may also be assessed. Sorting of query results is done by alphabetical order of organism by default, but users may also sort results by gene cassette array or *intI* genes.

Results obtained by using the *SEARCH* tool can be exported to a separate file in FASTA format allowing data to be used in further analysis.

The *BLAST* interface provides an alignment tool allowing the comparison of sequences submitted to the web site against the INTEGRALL database (Altschul *et al.*, 1997).

NOMENCLATURE guidelines and *GLOSSARY* of integron-associated genes are also present and aim to facilitate and standardize gene identification, as multiple names are often attributed to identical genes (for instance, in what concerns gene cassettes coding for aminoglycoside- and trimethoprim-resistance proteins).

The INTEGRALL platform also includes an external link to a discussion forum on integrons and other mobile genetic elements in order to promote communication and the exchange of ideas among scientists worldwide.

To avoid duplicate submissions, all entries are linked to the NCBI Genbank through its accession number; redundant GenBank entries are also eliminated. Regular updates are be performed to include in INTEGRALL newly released sequences.

6.3 The database

To date, INTEGRALL comprises more than 55 integron or integron-related nucleotide sequences and this number is continually increasing. Up to 69% of integron-related sequences correspond to uncultured bacteria and 28% were identified in the genomes of organisms belonging to the γ-Proteobacteria (**Figure 7.2**). The remaining 3% include a few bacterial representatives of α-, β-, δ- and ε-Proteobacteria, as well as Actinobacteria, Firmicutes, Cyanobacteria, Chlamydiae/Verrucomicrobia group, Bacteroidetes/Chlorobi group, Spirochaetes and Planctomycetes. Thus, integrons appear to have a broad-host-range (**Figure 7.3**), despite their higher prevalence in γ-Proteobacteria, which might be related to a higher number of studies of integrons in clinical settings comparing to natural environments.

Ecology of integron-carrying microorganisms, and thus environmental conditions that allow their dispersal, is also remarkably diverse: it includes wastewaters, river and marine waters, deep-sea vents, contaminated mine tailings, manure, soil and sediments, livestock and wild animals, food and clinical samples.

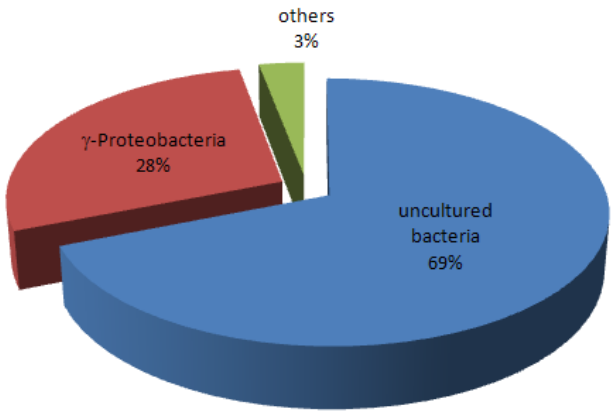


Figure 7.2 Host distribution of integron-related sequences deposited at INTEGRALL database (*n*=5573).

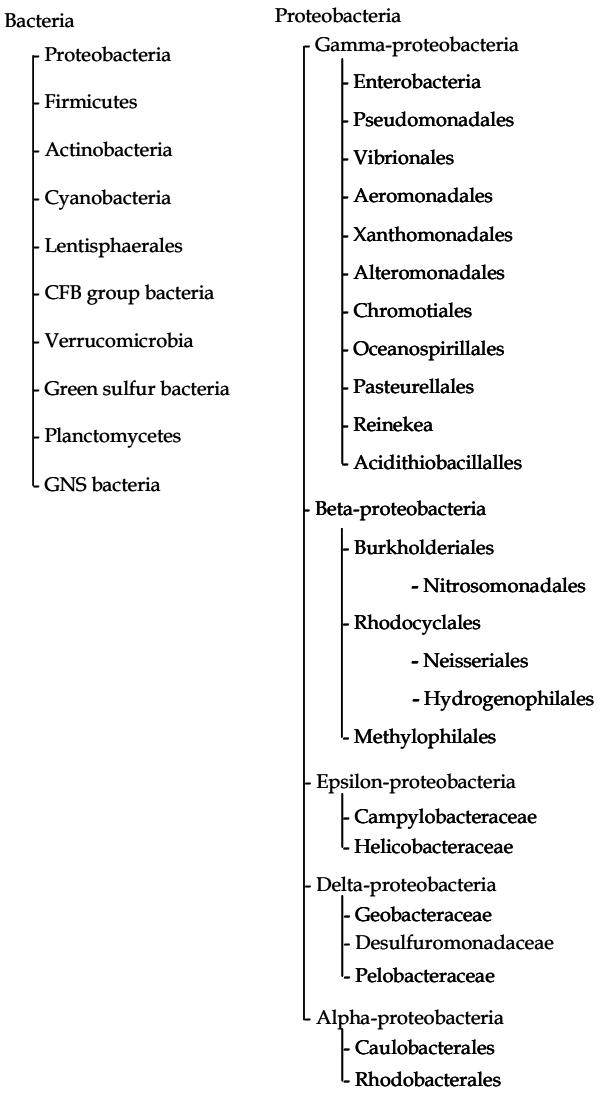


Figure 7.3 Host range of integron-related sequences deposited at INTEGRALL database.

Regarding the gene cassette pool, an important part of it is strongly linked to antibiotic resistance: 51% of gene cassettes reported encode antibiotic-resistance proteins (**Figure 7.4**). The most frequent gene cassettes encoding antibiotic-resistance proteins, confer resistance to aminoglycosides (54%), β-lactams (19%) and trimethoprim (19%) and chloramphenicol (6%) (**Figure 7.5**).

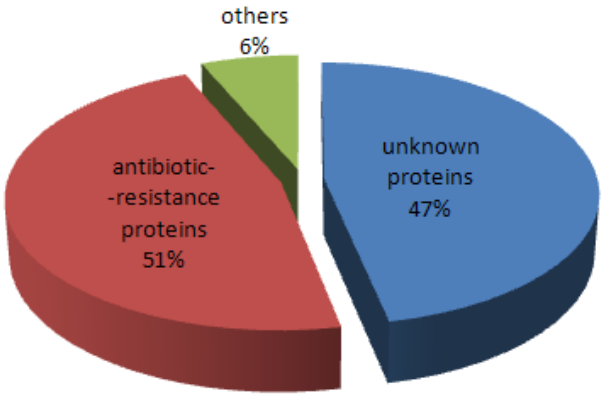


Figure 7.4 Distribution of gene cassette sequences deposited at INTEGRALL database (*n*=7150).

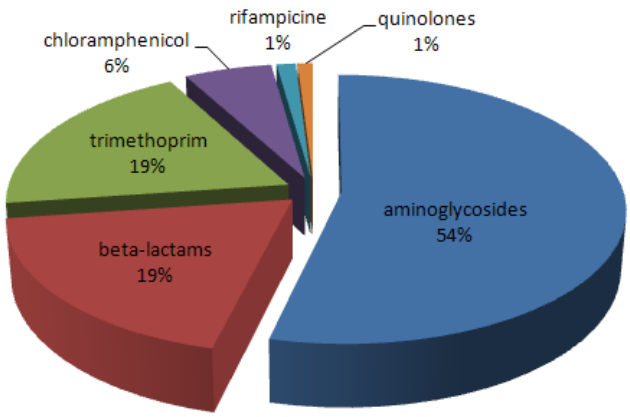


Figure 7.5 Distribution of gene cassettes coding antibiotic resistance proteins (*n*=3348).

In parallel, 47% of gene cassettes, mostly recovered from genetic libraries constructed using culture-independent methods, potentially encode proteins with unknown functions (**Figure 7.4**).

Studies are needed to elucidate these functions and whether the mobilisation and transfer of these genes may contribute to the fitness of bacterial strains in certain environments thus playing an important role in the adaptation and evolution of bacterial communities.

Future releases of INTEGRALL database and web-site will include the annotation of regulatory elements involved in integrase and gene cassette expression, and will allow indexing new gene cassette arrays with new integron names, in order to make of INTEGRALL the reference centre for integron data.

CHAPTER 8

General Discussion and Conclusions

8.1 Wastewater environments as reservoirs of integrons and gene cassettes

The use of treated effluents and sludge in irrigation and agriculture is a common practice in Portugal and worldwide. Reuse of treated wastewater (also referred as reclaimed wastewater) has enormous potential in preventing the degradation of water quality and drawdown of ground water levels. In addition, the presence of high concentrations of nutrients in treated wastewater reduces the requirements for inorganic fertilizers and consequently crop production costs.

The ecological and economic advantages of treated effluents are thus evident. However, the lack of studies concerning the persistence of genetic determinants of harmful microbial traits leaves unclear potential public health risks resulting from the use of treated effluents. Therefore, before the number of treated wastewater applications continues to grow, more research is needed in what concerns to the type of contaminants and pathogens that may persist after wastewater treatment.

The work presented in this thesis aimed to contribute to the knowledge on the molecular diversity of integrons and the potential risks it represents, and to evaluate the role of wastewater treatment plants in eliminating integron-carrying bacteria. For that, the occurrence and diversity of integrons was evaluated in wastewater environments of animal and human origin. The methods here applied aimed to gather complementary bacterial cultivation-dependent and independent approaches in order to circumvent the limitations of both methodologies.

Despite the high number of studies concerning microbial phylogenetic and functional diversity in treatment systems during the last two decades, little attention has been given to the perpetuation of antibiotic resistance mechanisms through wastewaters. Integrons are genetic elements that play a preponderant role

in bacterial innovation, by mediating the acquisition and expression of gene cassettes, such as those encoding antibiotic resistance. Therefore, this study aimed to examine several different aspects of integron diversity in wastewater environments.

Molecular techniques based on total community DNA were used to explore the diversity of the gene cassette pool without the biases associated with cultivation of microorganisms, as reported in **Chapter 2**. Nucleotide sequences retrieved showed an enormous reservoir of functionally diverse gene cassette, mostly coding for proteins with low homology to those deposited in public databases. Similar findings have been reported in marine metagenomes (Elsaied *et al.*, 2007; Koenig *et al.*, 2008; Wright *et al.*, 2008; Rodríguez-Minguela *et al.*, 2009), suggesting that the gene cassette pool is vast and yet unknown, possible playing important cellular roles that greatly go beyond antibiotic resistance.

Cultivation-independent approaches have the advantage to provide a better picture of total bacterial community structure and to access the entire mobile gene pool. Despite the advantages to assess gene diversity regardless bacterial host culturability under laboratory conditions, this cultivation-independent strategy clearly imposed restrictions on further identification of genetic contexts and bacterial hosts. Therefore, this was complemented with isolation of integron-carrying bacteria which, despite the bias introduced by the reduced culturability of environmental strains, allowed the identification of bacterial hosts and characterization of resistance phenotypes they display. This also constituted a first approach to assess the role of wastewater isolates as potential vectors of dissemination of integrons into natural ecosystems. As reported in **Chapters 3** and **4**, *Enterobacteriaceae* and *Aeromonas* spp. were used as bacterial indicators, since members of these families are frequently isolated in aquatic environments including sewage, seawater and freshwater ecosystems (Goñi-Urriza *et al.*, 2000; Henriques *et al.*, 2006; Jacobs & Chenia, 2007; Laroche *et al.*, 2009). Moreover, both contain important pathogens associated with human and animal infections (Linton & Hinton,1988; Khashe *et al.*, 1996; Fernandes *et al.*, 2008; Parker & Shaw, 2011). In

this work, the results obtained using the culture-dependent approach revealed a remarkable prevalence of integrons in animal effluents (~35%), almost all containing gene cassettes coding for antibiotic resistance. Besides erythromycin and ampicillin resistances (common in these taxa), increased resistances to streptomycin, trimethoprim-sulphamethoxazole and tetracycline were also observed (**Chapter 3**). In a recent European survey, these antibiotics have been reported among the most used in veterinary and animal farming (Grave *et al.*, 2010). Therefore these prevalences may result from the overuse of antibiotics, not only as therapeutics, but also as growth promoters and prophylactics in livestock. Other studies have also reported high prevalences of integrons in commensal bacteria, manure and amended soil (Binh *et al.*, 2009; Rodríguez-Minguela *et al.*, 2009; Yang *et al.*, 2010), strengthening the important role of farm animals in serving as reservoirs of integron-carrying bacteria.

Comparing to slaughterhouse's wastewaters, prevalence of integrons was lower in urban effluents (~3.7%), as reported in **Chapter 4**. Nevertheless, a higher diversity of gene cassette arrays was found in urban effluent, and included genes coding for extended-spectrum β-lactamases and fluoroquinolones. In addition, stronger promoters variants were also present in urban wastewaters (**Chapter 5**). As a consequence of these, integron-positive strains displaying higher levels of resistance to β-lactams (including 3rd generation cefalosporines), quinolones and fluoroquinoles were detected (**Chapter 4**). Thus, the efficiency of antibiotics recently introduced in human therapeutics can be compromised by resistance mechanisms associated to integrons. The presence of integron-carrying bacteria harbouring such traits in water environments poses an important public health concern: water is a good vehicle of dissemination, facilitating the contact of bacterial cells with human and other animals and eventually promoting its introduction into food chain. Previous studies focusing on comparative genomics of accessory modules located on plasmids from WWTPs have also suggested the existence of a common pool of resistance determinants shared among animal,

human and plant pathogens and other bacteria isolated from different habitats (Schlüter *et al*., 2007).

Taken together, results obtained with both complementary approaches suggest that despite most of the integrons found in *Aeromonas* spp. and *Enterobacteriaceae* strains were almost exclusively linked with antibiotic resistance, thus constituting a public risk concern, integrons may also encode other proteins. We hypothesise that the persistence of such gene cassettes, likely to impose an energy cost for the host, may be an indication of their possible contribution to environmental fitness. Increasing evidences have also been gathered by other authors based on mechanism of regulation of integrase expression that do place integrons as important players of cellular processes, such as SOS response (Guerin *et al*., 2009; Loot *et al*., 2010).

Although rarely recovered using culture-based techniques and/or using Gram-negative bacteria as target species, which seem to favour the retrieval of clinical-related integrons, the work reported here allowed the identification of novel putative encoding genes in *Aeromonas* strains. Of particular interest is the *dcyA* gene cassette identified in *A. caviae* ER.1.20 (**Chapter 4**), coding for a putative protein that, on the basis of homology analysis, is predictably involved in the promotion processes of cell interaction such as biofilm formation, by controlling the level of the second messenger cyclic dinucleotide c-di-GMP (D'Argenio & Miller, 2004). Moreover, recent findings support that c-di-GMP signalling plays a critical and possibly widespread role in bacterial virulence behaviour (Schirmer & Jenal, 2009). This may establish a link between integrons and virulence, enlarging the current knowledge of the diversity of class 1 integron gene cassettes in Gram-negative bacteria. Further investigation will allow understanding the specific role of DcyA as well as its dissemination among environmental and clinical bacteria.

8.2 Plasmid-mediated gene transfer in wastewater environments

As integrons cannot catalyse their transposition, their close association with transposons, IS and conjugative plasmids facilitates their dissemination through horizontal gene transfer. In the work here presented, the association between integrons and plasmids, in particular those belonging to IncP-1, IncN, IncW and IncQ groups was also investigated (**Chapter 2; Chapter 6**). Despite their capability of transfer to a broad-host-range, no positive detection for either of the focused groups was obtained among *intI*-positive strains used in this work (**Chapter 6**). Further investigations with probes targeting other replicon incompatibility groups (such as IncU and IncA/C, frequently reported in *Enterobacteriaceae* and *Aeromonas* spp. hosts) may allow allocating plasmids to specific incompatibility groups and to elucidate the diversity of plasmids present in these environments.

Nevertheless, broad-host plasmid replicons were detected in total community DNA revealing the prevalence of different groups among both treatment plants (**Chapter 2**). Previous studies based on exogenous plasmid isolation as well as on metagenomic analysis, showed the presence of IncP-1 and IncQ-like plasmids in urban WWTPs (Dröge *et al.*, 2000; Schlüter *et al.*, 2007; Schlüter *et al.*, 2008). Comparative genomics of plasmid accessory modules further revealed that animal and human pathogens share a common pool of resistance determinants, confirming the significant contribution of broad-host-range plasmids to the worldwide dissemination of integrons and many different resistance genes in bacteria from animal and human sources (Schlüter *et al.*, 2007). In this work, slaughterhouse wastewaters were found to be richer in IncP-1α, IncN, IncW and IncQ plasmids, while IncP-1β and IncP-1ε, IncQ were prevalent in urban wastewaters. Differences may be due to the selective pressures that shape the composition of bacterial communities in these wastewaters, since besides antibiotic resistance genes, plasmids can also carry genes involved in a wide range of metabolic activities, enabling bacteria to degrade pollutant

compounds. Future fully sequencing analysis of selected plasmids retrieved in this study will allow the identification of plasmid-borne integron genetic contexts.

8.3 Integron elimination and future directions on wastewater treatment

The findings reported in this thesis also showed that wastewater treatment is not effective in eradicating integron-carrying bacteria in final effluents, although national environmental quality requirements for effluent disposal were fulfilled.

In slaughterhouse's treatment plant, comparing to raw waters, an increase of integron prevalence was observed along the different treatment stages, with particular incidence at aeration tank (**Chapter 3**). Oxidation of organic matter and flocculation in aeration tanks enabled the breakdown and precipitation of biomass resulting in a reduction of up to 98% in the organic load of slaughterhouse wastewaters. However, the formation of bacterial aggregates, known to contribute to HGT events (Sørensen *et al.*, 2005), could explain this increased prevalence observed in treated effluent.

In urban wastewaters, a decrease on integron prevalence along treatment stages was observed, with exception for final effluent, where prevalence of *intI*-positive bacteria was higher comparing to those from aeration tank (**Chapter 4**).

Taken together these results indicate that, although secondary treatment shows efficiency on elimination of organic content, it is not effective in eliminating potentially harmful genetic traits such as integrons and integron-carrying bacteria.

Also noteworthy was the reduction of *intI*-prevalence in sludge recirculation in both urban and slaughterhouse treatment systems, suggesting that longer retention times and/or lower O_2 concentrations may contribute to the elimination of integron-carrying bacteria. These results are in accordance to those obtained in lab-scale reactors testing different digestion conditions for sewage sludge stabilization (Diehl & LaPara, 2009; Gosh *et al.*, 2009). In those studies, the use of aerobic digesters and shorter mean hydraulic residence times, as well as

mesophilic conditions, were generally ineffective in significantly decreasing *intI1* abundance (Diehl & LaPara, 2009; Gosh *et al.*, 2009).

Future analysis based on quantification of *intI* genes by qPCR should also be performed in field-scale studies and extended to other types of effluents, in order to evaluate the extent of this situation.

The presence of plasmid-borne integrons and antibiotic-resistance gene cassettes in treated effluents represents a risk for transfer of resistance genes to indigenous environmental bacteria, and eventually to animal and human gut commensals through consumption of agricultural products. Therefore, despite the costs associated to the implementation of tertiary treatment in wastewater treatment plants, this should be recommended in order to achieve complete elimination of bacteria. Moreover, tertiary treatment procedures can also contribute to eliminate antibiotics from final effluents, since photodegradation and chlorination, have been shown to successfully reduce tetracyclines, fluoroquinolones, β-lactams and trimethoprim concentrations in wastewaters prior to effluent release into natural aquatic environments (Macauley *et al.*, 2006; Dodd & Huang, 2007; Li *et al.*, 2008). Such approaches will allow to minimize the spread of integrons and the risk of transfer of resistance genes that can be perpetuated into aquatic and soil environments in downstream applications, being promising solutions for effective nutrient and microbial removal in wastewater treatment systems.

8.4 The need to congregate integron information

The goal of this thesis was to get insights into the diversity of the integron pool present in wastewater effluents. Due to the need of integrating integron structure, diversity and ecology, as well as genomic location, the INTEGRALL database was implemented as an effort to compile information on sequence data, integron ecology and standardize nomenclature (**Chapter 7**). INTEGRALL also

provides a compiled and manually curated sequence depository and a discussion centre for researchers. With the increasing number of sequences available at GenBank, the construction of dedicated databases has becoming a growing necessity to deal with structures with high degree of mobility and/or sequence divergence. The continuous grow and development of INTEGRALL may help to elucidate how integron diversity, gene cassette and integrase regulation may correlate with environmental niches and bacterial hosts. Ultimately, it will be used to clarify the dynamics and spreading fluxes of integrons between clinical settings, man-made systems and natural environments.

8.5 Summary of conclusions

In summary, the data presented in this thesis has provided a significant contribution to the knowledge about integron prevalence and diversity in wastewaters, encompassing a comprehensive study of integrons and their associations with other plasmid-encoded factors.

A significant contribution has also been made to the knowledge on the diversity of integron structures in *Enterobacteriaceae* and *Aeromonas* spp. outside clinical environments. In addition, the development, implementation and continuous curation of the INTEGRALL database facilitates the congregation of molecular integron information, contributing to the development of novel primers and probes and to the establishment of a reference centre for integron data.

Based on the results obtained in the course of this work, several conclusions can be drawn:

- the presence of new integrons, including in the treated effluent, draws attention to wastewaters, and in particular to domestic effluents, as sources and reservoirs of novel integron structures;

- the type of effluent affects both abundance and molecular diversity of gene cassettes: higher prevalence but lower molecular diversity of integrons were found in wastewaters from animal origin;

- in both WWTPs, gene cassette expression was mainly controlled by weaker promoter variants (PcW and PcH1), thus compensating a lower expression efficiency with a higher efficiency for gene cassette rearrangements;

- integron-associated gene cassettes retrieved may play an important role in bacterial adaptation to the intensive selective pressures characteristic of these environments;

- wastewaters constitute potential hot spots for horizontal gene transfer, integron dissemination and selection of antibiotic resistance genes, emphasizing the urgent need to employ effective means of effluent disinfection in wastewater treatment plants.

8.6 Future perspectives

The study presented here raises a number of questions on the role of integrons in bacterial adaptation as well as on the concern to avoid spreading of integrons and antibiotic-resistant bacteria into natural ecosystems.

Further studies will continue to contribute to the understanding of the role of integrons in bacterial fitness and will also allow elucidating the vectors involved in their spreading. Thus, it is our aim:

- To elucidate the role of novel gene cassettes identified in this study, in particular the putative role of *dcyA* in cell signalling and bacterial adaptation;

- To extend integron-borne plasmid characterization to more incompatibility groups, in order to elucidate mechanisms of transmission and the ecology of plasmid-borne integrons;

- To extend integron detection and characterization to more bacterial groups, in order to compare the prevalence and diversity of integron structures found in this study with those from other *taxa*;

- To investigate the effect of tertiary treatment on the elimination of integron-carrying bacteria in final effluents, to avoid their spreading and perpetuation in natural environments;

- To continue to develop INTEGRALL database in order to include more information on integron data and tools to help to elucidate integron regulation and ecology.

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