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Minireview

# Molecular biology of class 1 mobile integrons

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**Summary.** The rapid spread of antibiotic resistance has significantly limited treatment options, increased mortality rates of infections and has become a major clinical and public health problem. Integrons are genetic platforms carried by plasmids or contained within a transposon, and their role in the dissemination of resistance genes among bacteria has been well established and documented. Mobile integrons of class 1 are the most ubiquitous and have been the most commonly reported among clinical bacteria and are predominantly associated with Gram-negative bacterial pathogens. Although there is a number of currently available studies of class 1 integrons, only a limited number of processes crucial to the understanding of integron biology have been elucidated. Among these processes are the molecular mechanism of integrase gene expression as well as gene cassette expression in different bacterial pathogens.

**Keywords:** antibiotic resistance, gene expression, horizontal gene transfer, mobile integrons, recombination.

Antibiotics have transformed modern medicine, reducing childhood mortality and increasing life expectancy, and they have played a pivotal role in the major advances in medicine and surgery (Lee Ventola 2015). Thus, their discovery is considered a turning point in human history. The successful use of antibiotics is nowadays compromised by the potential development of tolerance or resistance to these drugs. Regrettably, the use of antibiotics has been accompanied by the rapid development and dissemination of resistant bacterial strains, and this scenario has been seen in nearly all antibiotics that have been developed (Lee Ventola 2015). Antibiotic resistance is a natural phenomenon, and bacteria constantly evolve, acquiring resistance to natural antibacterial products; however human activity has changed the selective pressure of antibiotics due to overuse and misuse. Human medicine is not the only creator of the evolutionary pressure for the emergence of antibiotic resistance; also involved is the use of antibiotics in animal treatment and growth promotion, horticulture, beekeeping, antifouling paints (used in the marine and oil industries) and in laboratories carrying out genetic manipulations (Nsofor 2016). Due to the phenomenon of antibiotic resistance, the number of infections caused by multidrug-resistant bacteria is increasing globally and clinical failures in bacterial-mediated diseases have been recognized as a reality of modern society (Gross 2013; Spellberg and Gilbert 2014). Thus, many public health organizations have recognized that we are now in a "post-antibiotic era" and warn that the antibiotic resistance crisis is becoming dire (Michael et al. 2014).

Bacteria can be intrinsically resistant to certain antibiotics but can also acquire resistance to antibiotics through mutations in chromosomal genes and by means of horizontal gene transfer. While mutation events contribute to bacterial adaptation, horizontal gene transfer seems to be the major contributor to the emergence, recombination and dissemination of antibiotic-resistance genes across a wide diversity of bacteria, including clinically significant bacterial pathogens (von Wintersdorff 2016). The acquisition of resistance genes via horizontal transfer involves mobile genetic elements such as plasmids and transposons. Integrons are mostly carried by plasmids or contained within a transposon. The role of integrons in the dissemination of resistance genes among bacteria has been well established and documented (Sundström 1998).

Integrons are genetic platforms that allow bacteria to evolve rapidly through the acquisition, stockpiling, excision

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and reordering of open reading frames (ORFs) found in mobile elements called cassettes (Fig. 1). The acquisition of gene cassettes is mediated by site-specific recombination, followed by the conversion of cassettes to functional genes through expression driven by integron promoters (Stokes and Hall 1989). The evolutionary potency that integrons provide for bacteria is based on the variety of functions encoded in the cassettes, as well as on the intricate coupling of integron activity to bacterial stress (Stokes and Hall 1989). The structure of any integron includes a stable platform and a variable cassette array. Integrons consist of three elements: the gene that encodes an integrase (tyrosine recombinase, encoded by the intI gene) needed for site-specific recombination within the integron; the primary recombination site (*attI*) that is recognized by the integrase; and the promoter (Pc), located upstream of the integration site, necessary for the efficient transcription and expression of gene cassettes present in the integron (Fig. 1). In particular, integrons as genetic elements exhibit two types of genomic innovation: integration of novel genetic material without disruption of pre-existing genes (without insertional inactivation) and expression of integrated ORFs that enables immediate natural selection. Integrons are traditionally divided into mobile integrons and chromosomal (sedentary) integrons based on their context. Division of integrons into these two groups still exists in the literature although it is known that there is a genetic continuum of structurally different integrons between these groups (Hall et al. 2004, 2007; Boucher et al. 2007). It is now broadly accepted that mobile integrons evolved from chromosomal integrons that found their way into clinically relevant bacteria through association with transposable elements and conjugative plasmids (Mazel 2006; Boucher et al. 2007; Cambray et al. 2010). Most mobile integrons have been described in Gram-negative bacteria, and only sporadically in Gram-positive bacteria (Martin et al. 1990; Nesvera et al. 1998; Nandi et al. 2004; Shi et al. 2006; Xu et al. 2010; Barraud et al. 2011). Based on the genetic relatedness of the integrase *intI* gene sequence, the mobile integrons were initially classified and divided into five classes. However, extensive DNA sequencing efforts have further revealed a broader genetic diversity of the integrase gene and identified >90 different gene variants, thus questioning the initial classification scheme (Boucher 2007; Gillings 2008). Most of the currently available studies on integrons had been conducted on class 1 integrons, since the class 1 integron platform is the most ubiquitous and has been the most commonly reported among clinical bacteria, with a focus on Gram-negative bacteria. Due to their clinical significance, mobile class 1 integrons are the subject of this review.

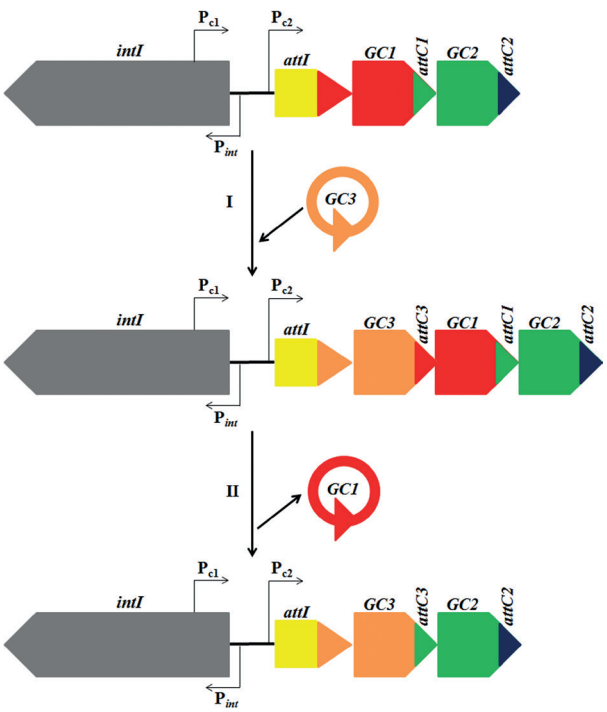


**Fig. 1**. Functional elements of a mobile integron class 1 and schematic presentation of gene cassette integration and excision. *intI* – integrase gene, $P_{c1}$ and $P_{c2}$ – promoters directing transcription of gene cassettes, $P_{intI}$ – promoter of the integrase gene, *attI* – primary recombination site of integron, *attC* – gene cassette recombination site. I – integration of new gene cassette, II – excision of a gene cassette.

## Functional elements of class 1 mobile integrons involved in cassette shuffling

The primary recombination site of class 1 integrons, *attI*, in which gene cassettes are integrated, is composed of two binding sites for integrase, L and R. The recombination site (5'-GTT-3') is located within the R, and the recombination event occurs between the A and C nucleotides of the complementary strand, which is designated as the bottom strand in the biology of integrons. Additionally, within *attI* and upstream of L and R, two imperfect direct repeats are present, DR1 and DR2, which are additional binding sites for integrase and function as a molecular trap whose biological function is to keep integrase monomers close to the recombination site (Gravel et al. 1998).

Integron cassettes constitute the variable and mobile part of the integron. Gene cassettes typically consist of an ORF devoid of a promoter, closely bounded by a multifunction site termed *attC*. The *attC site* is analogous to *attI* of the integron, and serves as both an integrase recognition and a recombination site. Since typical integron cassette carry a single ORF, they are comparatively small, usually between 500 and 1000 bp. Integron cassettes can be found in a linear double-stranded form as part of an array within an integron, or, when excised, as a free, nonreplicative circular element that can move between integrons. Cassette arrays of integrons can vary in length and those within class 1 integrons are typically short (1-8 cassettes) arrays of antibiotic resistance genetic determinants (Naas et al. 2001).

The *attC* site is an integral part of gene cassettes that is necessary for their mobility by integrase-mediated integration or excision. These sites differ significantly from canonical tyrosine recombinase sites. A common feature of all *attC* are regions of inverted homology designated as R"-L" and R'-L' separated by a region of varying length. An important characteristic of *attC* is the lack of conserved nucleotide homology, which is restricted only to 5'-GTT-3' triplets (Stokes et al. 1997). Nevertheless, *attC* has a conserved palindrome organization that enables the forming of secondary structures during intramolecular pairing in the DNA (Hall et al. 1991). When secondary structures within these regions are formed, they resemble canonical L and R sites of *attI*. These secondary structures within single-strand DNA are recognized by integrase. Thus, genetic information necessary for recombination in *attC* is not determined by the primary nucleotide sequence but instead by the characteristics of the secondary structures (Bouvier et al. 2005). The ability of bacteria to recombine incoming gene cassettes based on structural recognition elements instead of sequence information *per se* greatly enhances their ability to adapt to changing environmental conditions.

Integron integrases belong to the family of site-specific Y recombinases, but form a specific subfamily within it (Nunes-Düby et al. 1998). It is generally accepted that Y recombinases process the recombination between two DNA molecules in a set of archetypal steps. The process starts with the assembly of the synaptic complex, which is formed by the two DNA substrates and four monomers of the recombinase protein. It then proceeds through the exchange of a set of strands from each partner molecule, forming a transient Holliday junction, and ends with the resolution of the junction through a second strand exchange. However, integrase is an atypical tyrosine recombinase from a structural point of view (MacDonald et al. 2006). It processes recombination between double-stranded (*attI*) and single-stranded (*attC*) substrates. Additionally, it can process recombination between two double-stranded substrates (*attI*x*attI*) or two single-stranded substrates (*attC*x*attC*). This poses a mechanistic problem, since when one substrate is single-stranded the first strand exchange generates an asymmetric, atypical Holliday junction that cannot be resolved through the classical second strand exchange (MacDonald et al. 2006; Loot et al. 2012). Details of recombination mechanisms will be explained in detail further in the text.

## Recombination reactions

As previously stated, integrase recognizes different recombination sites and enables five different recombination events: *attI* x *attC*, *attC* x *attC*, *attI* x *attI*, *attI* x non-specific secondary sites with GTT and *attC* x non-specific secondary sites with GTT. The recombination event *attI* x *attC* is the most efficient, while *attI* x *attI* is $10^3$ times less efficient (Bouvier et al. 2005). Having in mind that recombination events involving *attC* include single-stranded, but structured DNA, it is hypothesized that these events occur not only during natural transformation and conjugation, but also during transcription, DNA repair or replication (Baharoglu et al. 2010, 2012). Additionally, structural disturbances of double-stranded DNA resulting in cruciform structures have been detected in bacterial cells during specific stress conditions.

The recombination event that results in the integration of gene cassettes takes place between double-stranded *attI* and single-stranded *attC*. Since it generates an atypical Holliday junction it cannot be resolved by secondary strand exchange. Instead, resolution takes place by means of replication (Loot et al. 2012). However, it is still unclear whether it is a passive process where replication is driven by a replisome formed within the origin of replication (*oriC* or *oriV*) or by an *ad hoc*-formed replisome. After the incision of the gene cassette, its stability depends on selection. There are three possible results of integration if we consider that integration events are subjected to selection: (i) if a gene cassette enables selective advantage to bacteria it is then subjected to positive selection; (ii) if a novel gene cassette is toxic or does not provide selective advantage to the cell but with its integration lowers the expression of cassettes enabling selective advantage by distancing them from the promoter, selection is against such a cassette; (iii) if a gene cassette does not affect phenotype, the change is neutral and further recombination dynamics depend on environmental variations.

A recombination event between two *attC* sites located within the same array of gene cassettes results in the excision of integron cassettes (Collis and Hall 1992a, 1992b). Two single-stranded folded *attC* sites must be recruited at the same time by the integrase to generate, after the first strand exchange, the Holliday junction. As described previously, the Holliday junction is atypical and resolution takes place semi-conservatively, by means of replication (Loot et al. 2012). This means that replication of the recombined bottom strand releases a covalently closed single-stranded cassette and the original DNA molecule lacking the excised cassette, while the replication of the nonrecombined top strand would reconstitute the initial substrate (Loot et al. 2012). After the excision, the gene cassette can be integrated again in the same integron, and this reaction is favored due to the efficiency of the *attI* x *attC* recombination. The biological significance of such an event is the reuse of cassettes that were silenced due to distance from the promoter. Nevertheless, excised cassettes can be degraded by the cell factors of the host bacteria.

## Expression of the mobile class 1 integron elements

Regulation of the expression of gene cassettes within an integron takes place both at the level of transcription and of translation (Collis and hall 1995; Jové et al. 2010). As the genes in integron cassettes are generally promoterless, their

expression is ensured only when inserted into an integron at the *attI* site by the proximity of the external Pc1 promoter located within the integrase gene or by the Pc2 promoter located in the vicinity of the *attI* site. The Pc2 promoter is usually inactive due to the improper distance between -35 and -10 elements (Jové et al. 2010) (Fig. 1). However, when this distance is canonical and Pc2 is active, Pc1 is usually very weak. Variations in the Pc1 promoter strength affect the protein sequence of the integrase, resulting in three major alleles of integrase class 1 that have similar integration activity but differ significantly in efficiency of gene cassette excision (Jové et al. 2010). Thus, a correlation has been established between Pc1 promoter strength and the efficiency of integrase; integrase is the most efficient when Pc1 is the weakest. The length of transcripts from Pc promoters varies and a transcript usually encompasses only several cassettes. For some time it was thought that the structure of *attC* affects the length, since *attC* can function as a Rho-independent terminator, although it does not have a T-rich region after the hairpin that is the hallmark of these terminators. It is worth noting that, although rare, integron cassettes sometimes harbor their own promoter. The expression of these integron cassettes is assured, regardless of their position in the array.

The expression of integron cassettes is also governed by the presence of a binding motif that initiates ribosome assembly (Hanau-Berçot et al. 2002; Jacquier et al. 2009). Gene cassettes that do not have a ribosomal binding site within their transcripts rely on upstream ORF for initiation of translation (Hanau-Berçot et al. 2002). All class 1 integrons have short *orf11*, ahead of which the ribosome-binding site located in *attI* is located, and are thus present in all polycistronic transcripts originating from the Pc1 promoter (Hanau-Berçot et al. 2002). The position of *orf11* overlaps with the recombination point, resulting in a variable 3' end that depends on the first cassette of the array.

Like all elements promoting genetic variation in a cell, integrons must be well controlled to avoid the deleterious effects of an overwhelming recombination activity. Despite the well-documented role of class I integrons in the rapid dissemination of antibiotic resistance, the dynamics of cassette recombination that depend on integrase gene expression have not been well-enough explained as yet. It was shown that within the promoter of the *intI* gene LexA, a binding motif is present and it overlaps with the -10 element of the promoter (Cambray et al. 2011). The LexA protein is a transcriptional regulator that represses the SOS response of bacteria by allosteric interference with the RNA polymerase (Walker 1984). The SOS response system enables DNA repair and is associated with a number of clinically significant phenotypes like the multiplication of resistance alleles due to hypermutagenesis and the activation and dissemination of virulence factors associated with bacteriophages, transposons and other genetic elements. Recently it was demonstrated that some antibiotics such as β-lactams, fluoroquinolones and trimethoprim can induce a SOS response in bacteria (Phillips et al. 1987; Miller et al. 2004; Kelley 2006; Erill et al. 2007). When the SOS response is activated, LexA undergoes autoproteolytic inactivation and *intI* gene expression is activated. Induction of integrase expression leads to an increase in recombination event frequency resulting in integration or excision of the gene cassettes. The biological meaning of coupling the SOS response and integrase expression emerges if we take into account bacterial physiology. This control allows a subtle coupling of integron activity with bacterial physiology. By linking the activity of the integron to an alarm signal, recombination events are limited to the times when bacteria need to evolve and adapt. During these moments gene cassette acquisition or reshuffling can have a dramatic impact on bacterial survival. On the other hand, repressing the expression of the integrase when a bacterial cell is well adapted is beneficial, because it prevents the reshuffling of cassettes when the configuration of the array is optimal and avoids random recombination events at secondary sites that could be deleterious. A matter that had remained elusive was the presence of cassettes containing genes that confer resistance against antibiotics that do not damage DNA and do not induce the SOS response in *Escherichia coli*. It led to the discovery that some antibiotics, such as aminoglycosides, activate the SOS response in species other than *E. coli* (Baharoglu and Mazel 2011). Also, it emphasized the importance of analyzing integrase expression in different bacterial species, especially in those of clinical importance. This approach allowed for the discovery of a still unknown level of control of integrase gene expression through the cAMP receptor protein (CRP), which has been identified in the chromosomal integron of *Vibrio cholerae* (Baharoglu et al. 2012). CRP is the master regulator of the carbon catabolite repression response, adapting cellular metabolism to the type of carbon sources available in the environment. In the case of *V. cholerae*, a CRP-binding box is present between the integrase promoter and Pc, thus controlling expression independently of the SOS response and connecting the integron to environmental conditions (Baharoglu et al. 2012). Additionally, it was demonstrated that some of the nucleoid proteins like Fis, IHF and H-NS, affect expression of the intI gene.

## Concluding remarks

Antimicrobial resistance remains the leading concern in global public health. It is considered that integrons play a core role in the antibiotic resistance of microorganisms and they have been shown to contribute to the wide spread and distribution of antibiotic-resistant genes among bacteria. Although there is a number of currently available studies of class 1 integrons and while a whole range of processes critical to our understanding of the biology of integrons has been elucidated, the integrons examined herein remain to be examined in more detail. The origin of gene cassettes has not

been identified as yet, and the creation of cassettes remains a subject of the utmost importance for understanding integrons, for which an indisputable model is not available. Additionally, published studies have revealed that the integron is intimately connected to bacterial physiology and the environment through the mechanisms of integrase expression regulation, and that this regulation could be species-specific. Therefore, further efforts for identifying novel integron regulatory pathways in clinically significant bacterial pathogens are needed.

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