Review Article:

Integrons and antimicrobial resistance in bacteria: A systematic review

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ABSTRACT

Resistance to antimicrobial agents is on the rise and this phenomenon not only leads to an increase in economic burden but may also cause serious therapeutic problems. Nowadays, it is known that horizontal transfer of resistance genes is a major cause for spreading antibiotic resistance genes in microbes. The previous studies have manifested that integrons play a significant role in horizontal transfer of antibiotic resistance. Integrons are in fact natural cloning and expression systems which have the ability to spread multi drug resistance (MDR) in bacteria. They are normally motionless but can be transferred through mobile genetic elements, for example plasmids and transposons. Integrons carry divergent gene cassettes that are rearranged under antibiotic selective pressure. It is based on the sequence of the integrase gene that various classes of integrons are known. Class 1 integron is the most prevalent type among bacteria. This review highlights the need for continuous surveillance to understand the dissemination of integron and multidrug resistance among different bacteria.

Key words: Integrons; Drug resistance; Bacteria

INTRODUCTION

Antibiotics are synthetic or natural components some of which can inhibit the bacterial growth while others can have lethal activities. Based on their selective acting, antibiotics are not harmful to humans, but are destructive to bacterial cells. While developing new drugs, the main reason for dissemination of antibiotic resistance in bacteria has been the overuse, underuse, and misuse of antibiotics. In fact, antibiotic resistance among micro-organism mediated infectious diseases is still among the great concerns for global public health [1]. Gradually, along with the development of new drugs, drug resistance problems expanded after their discovery in 1940. In the long run, the multi-resistant bacteria were found in 1950 as well. Therefore, an immediate and necessary need is felt to understand drug resistance mechanisms in multidrug resistant pathogens and to target these mechanisms (e.g., antibiotic target site mutation, efflux pump for antibiotic expulsion, etc.) so as to deal with these pathogens. Although the main mechanisms behind antibacterial resistance are now understood, new and significant features are being discovered. Nowadays, horizontal transfer of resistance genes is known as a major reason for spreading antibiotic resistance genes in microbes. Horizontal Gene Transfer (HGT) is the transfer of genetic material to bacteria from the same generation and a successful HGT relies on the introduction of DNA into a recipient cell's cytoplasm and heritability of the transferred sequences in the recipient microorganism. HGT consists of conjugation, transduction, and transformation with mobile genetic elements, like conjugative elements, insertion sequences, transposons, miniature inverted-repeat transposable elements, and integrons, as the major contributor in these genetic transformations, which are able to share genetic information between bacteria [2]. All these are

shown to be significant in resistance gene movement among clinically important bacteria. Furthermore, recombination mechanisms have proved to have a vital role in genetic mobility and acquisition of drug resistance. Recombination is categorized into different modes: 1) General or homologous recombination takes place among DNA molecules of very similar sequence, which often occurs during conjugation and replication in order to repair DNA damage in bacteria, and 2) Illegitimate or non-homologous recombination, which happens in regions where there is not any sequence resemblance, 3) Transposition recombination is the movement of a DNA element from one locus to another, which usually does not need homology among the recombining DNAs and comprises a discrete transposable element, and 4) Conservative site-specific recombination, which involves the recombination between short homologous sequence mediated by a particular enzymatic machinery, which is essentially one enzyme for each special site. The best example of site-specific recombination is the integration of bacteriophage Lambda (λ)[1]. Plasmids, transposons, and integrons are shown to cause horizontal gene transfer of antibiotic resistance genes [3]. According to the literature, it is documented that integron has a major link to acquisition, expression, and dissemination of antibiotic resistance genes while being able to do more, including having a strange aspect of antibiotic-resistant pathogens and possessing a main role in bacterial adaptation and genome evolution.

INTEGRON STRUCTURE

Integrons as a natural cloning and expression systems are efficient in capturing and expressing exogenous genes. All integrons have three essential features: 1) IntI, which encodes a tyrosine recombinase family integrase and mediates the recombination between incoming gene cassettes (attc); 2) Proximal primary recombination site, *attI*, and when gene cassettes is integrated, it is expressed by last feature: 3) integron-associated promoter, Pc [4]. In spite of integrative and conjugative elements in integrons, they are motionless. Several studies have shown the “clinical” integrons, borne on plasmids, are specific to pathogens, while chromosomal integrons are the norm for environmental bacteria. Meanwhile, a few classes of integrons, which are borne on conjugative plasmids like R388 (IncW group) and R46 (IncN group), are directly connected to conjugative transfer mechanisms [1].

The term "chromosomal integrons" (super integrons) was made and introduced by Mazel et al (1998) responding to the use of the term "mega-integron" by Clark et al (1997) [5, 6]. Mazel divided integrons in two significant groups: Floating integrons (also called Resistance Integrons -RI- or Multiresistance Integron (MRI) or mobile integrons (because of their link with transposons or plasmids for their mobility) and superintegrons. The superintegrons, which could have hundreds of cassettes have homogenous *attC* sites, giving them the ability to encode for different functions, like antibiotic resistance and resistance to disinfectants, while the mobile integrons, which have few cassettes, merely encode for resistance against one or (more commonly) various antibiotics. It is obvious that the essential elements of an integron are settled in 5’-conserved segment (CS), which can encode an integrase with a variable region of gene cassettes, located in 3’-end. A gene encoding integron integrase (*IntI*) is located in an adjacent recombination site (*attI*), where gene cassettes are integrated, and Pc, for the expression of these integrated gene cassettes. Gene cassettes are compact DNA elements with the simple structure, consisting of a recombination site and a single open reading frame. This basic structure can vary mainly in terms of the identity and orientation of the embedded open reading frame. A majority of cassettes involve a single ORF, oriented from left to right, while cassettes with two or more ORFs, no ORFs, or ORFs in reverse orientation are also known [7, 8]. There are differences in length and sequence of the of the *attC* sites of the gene cassettes (from 57 to 141 bp), while they are similar in the inverse core site (RYYYAAC) and the core site (GTTRRRY; R= purine, Y= pyrimidine). The homologous recombination between core-site sequences in the *attI* and *attC* sites results in the insertion of the gene cassette downstream of a resident promoter within the

integron that drives expression of the encoded product [4].

RECOMBINATION REACTIONS IN INTEGRON

Integron includes i) an integrase gene (*IntI*) which encodes a tyrosine recombinase (Y-recombinase) performing the site-specific recombination reaction, ii) a primary recombination site (*attI*) where the integration of gene cassettes occurs, and iii) a strong promoter (Pc). Gene cassettes correspond to open reading frame encoded by the cassette, and the *attC* site, which contain integrase binding domains R´, L´, L″, and R″. The length of natural *attC* sites varies from 57 to 141 bp. These elements have partial palindromic sequences, such that R´ can pair with R″ and L´ with L″, thus forming a constant cruciform structure recognized by integron integrases [9, 10]. An extra base, L″ ensures correct orientation and insertion of cassettes into the array [11]. Two recombinations occur in the following sequences: one cassette insertion mainly happens through intermolecular recombination between an *attC* and the *att*I site and the other cassette insertion principally occurs through intramolecular recombination between two *att*C sites in a cassette array excises cassettes as DNA circles, though less efficiently, compared with the former recombination. This means that insertion of cassettes into an array during attc*attc recombination is possible, and yet, attI is the preferred insertion point for incoming cassettes [12]. The recombination between *attI* and *attC* involves only the bottom strand of the incoming *attC*, and the single-stranded recombination structure is then resolved through replication [13]. The *attI* site, similar to *attC*, carries integrase binding sites, called L and R. The R binding site includes the canonical sequence 5″-GTTRRRY, with incoming gene cassettes being inserted between the G and T residues [14]. At first, only two opposite monomers are active and cleavage of one strand of each substrate by their nucleophilic tyrosine leads to initiation of recombination. Covalent phosphotyrosine bounds are formed between the attacking monomers and the 3´-ends of DNA, while the 5´-OH ends stay free. The following step includes the 5´-ends attacking the opposite 3´-phosphodiester bonds, ending in the first strand exchange, forming a Holliday Junction (HJ). The complex can then isomerize the inactive monomers, becoming active and vice versa. Then, a second strand exchange can proceed following the accurate same mechanism. This last strand transfer resolves the HJ, frees the proteins, and the recombination response is obtained [15].

After this recombination reaction, there comes the time for gene cassettes expression. Gene cassettes depend on an external promoter for expression which is usually located in *IntI* gene and *attI* in class 1, 2, and 3 integron. Promoters within the integron integrase gene drive expression of gene cassettes in the associated array, but when cassettes come to more distal to the promoter, the strength of expression decreases [16]. Some chromosomal cassettes are too long for expression of all the cassettes to be driven by a single promoter; however, these cassettes are transcriptionally silent or carry their own internal promoters [17]. Internal promoters identified in *cmlA* chloramphenicol resistance gene cassette [18] and toxin-antitoxin (TA) genes are a common example of large chromosomal cassette arrays and are believed to help array stability [19].

BIOLOGICAL FUNCTIONS RELATED TO INTEGRONS

Some studies indicated that superintegron gene cassettes encode proteins which are involved in pathogenicities, such as the heat-stable toxin gene (*sto*), the mannose-fucose-resistant haemagglutinin gene (*mrhA*), and a lipoprotein gene in *V. cholera [20]*. The capsular polysaccharide *V. vulnificus*, which is identified by transposon-mutagenesis screen, is present in a superintegron gene cassette as well [21]. Superintegron gene cassettes encode the metabolic functions of three proteins: a *V. cholera* sulphate-binding protein, a psychrophilic lipase in *Moritella marina*, and a restriction enzyme (*XbaI*) and its cognate methylase from *X. campestris* pathovar *badrii [22]*. Proteins have homology to DNA-modification enzymes (including nudix hydrolases and restriction

endonucleases) and enzymes involved in the primary metabolism are encoded by super integrons. Superintegrons carry several gene cassettes encoding members of different toxin–antitoxin families found in low-copy number plasmids that carry their own promoter and are expressed outside the context of *attI* and the Pc promoter control. They might have a significant effect on stabilizing the large arrangement of cassettes discovered in the superintegrons in *Vibrio spp [23]*. Cassette gene product is quite important in biofilm formation and interaction with the local environment [24]. Subsequently, it is deduced that integron and their associated gene cassettes are important components of bacterial adaptation and adaptive variation.

DIFFERENT TYPES OF INTEGRONS

Five classes of mobile integrons are known according to the homology of their integrase genes.

Class 1 Integron is commonly discovered in about 40 to 70% of gram-negative pathogens such as *Acinetobacter, Aeromonas, Alcaligenes, Burkholderia, Campylobacter, Citrobacter, Enterobacter, Escherichia, Klebsiella, Mycobacterium, Pseudomonas, Salmonella, Serratia, Shigella, and Vibrio,* occasionally in *Campylobacter jejuniand and etc,* with a broad distribution of 22-59% on their plasmids, which can be transferred by conjugation, and which can be embedded in larger transposons including Tn21 [25]. It contains an integrase (*int*), a recombination site (*attI*), and gene cassettes which are not part of integrons till they become integrated. Expression of the integron depends on the promoter (P_{ANT}) where it is located in 5'-conserved segment and is divided into two promoters: P1 and P2. 3'-conserved segment, which is not extensively defined includes a Δ*qacE* (encoding an efflux mechanism which mediate resistance to quaternary ammonium compounds), a *sulI* gene (encoding dihydropteroate synthase causing resistance to sulfonamides), and *ORF5* of unknown function. Currently, class 1 integrons are the most conventional types of integrons which can be found in clinical isolates such as the *Enterobacteriaceae.* The most identified gene cassettes among *Enterobacteriaceae* are those encoding resistance to streptomycin (*aadA*) and trimethoprim (*dfrA*] [26]. Two new resistance cassettes which encode novel carbenicillinases - CARB-7 and CARB-9 - have been identified in the super integrons of two environmental isolates of *V. cholera* that confer various levels of resistance to β-lactam antibiotics. These novel carbenicillinases are members of a subgroup of the class A of β-lactamase family, the RSG carbenicillinases, which are connected with either a VCR-like *attC* site of 123 bp or an *attC* site of 103 bp, which have been recognized in class 1 integrons from *V. cholera, Pseudomonas aeruginosa, Proteus mirabilis*, and *Salmonella enterica* serovar *Typhimurium [27]*. In general, there are over 130 known various antibiotic resistance gene cassettes from clinical class 1 integrons, together with a few number of other gene cassettes of unknown functions [28] which are common in the pathogens and commensal flora of livestock and companion animals [29]. They have also shown to have made their way into plant pathogens [30] and Gram-positive organisms [31].

Class 2 Integron is embedded in the Tn7 family of transposons and its derivatives (Tn1825, Tn1826, and Tn4132) with their gene cassettes containing the *intI*2 gene. This gene commonly contains a nonsense mutation in codon 179 that produces a nonfunctional protein, including a termination codon. Since the integron integrase gene of clinical class 2 integrons is inactive, it is not surprising that their cassette arrays are highly conserved [32]. The 3' segment of Class 2 integrons contains five tns genes (*tnsA, tnsB, tnsC, tnsD*, and *tnsE)*; they are responsible for two recombinational pathways: in the main transcription pathway, the transposition is under control of *tnsD*, which is known as a particular sequence called *att*Tn7, discovered in the chromosomes of various bacteria; however, the mobilization of Tn7 is not destructive since the insertion is downstream of the region encoding the *glmS* gene and the other pathway of the transposition is led to conjugative plasmids and to the genomes of filamentous bacteriophages [25, 33]. Low variety of integrated gene cassettes and most reported class 2 integrons carry 3 particular and conserved gene cassettes: dihydrofolate

reductase (*dfrA1)*, which confers resistance to trimethoprim, streptothricin acetyltransferase*(sat2)*, which confers resistance to streptothricin, and aminoglycoside adenyltransferase *(aadA1)*, which confers resistance to spectinomycin and streptomycin [32]. *OrfX*, which carries a truncated *attI2* site, corresponds to the *ybeA* gene. There is a region between the *OrfX* and the *tnsE* genes which contains three additional genes (*ybfA*, *ybfB, and ybgA*). Moreover, there are two new resistant determinants in Class 2 integrons. The sequence analysis represented that downstream of the stop codon of the *catB2* gene, there is a 259-bp region with 100% homology to the *attI2* class 2 integron recombination site, which can be replaced by the typical *attC* site. Therefore, the novel integron structure class 2 is *sat2-aadB-catB2 (ΔattC)-dfrA1-sat2-aadA1-orfX* [25, 33, 34]. Similar to class 1 clinical integrons, class 2 integrons from clinical contexts have made their way into the different kinds of pathogens, commensals, and environmental microorganisms [34].

Class 3 Integron, isolated from a carbapenem-resistant *Serratia marcescens* strain was characterized by Collis et al [33]. They demonstrated that the integron module consists of the *intI3* gene, *attI3* site, and Pc promoter. Among the β-lactamase gene cassettes, bla_{VEB-1}, was the first to be recognized as encoding a class A enzyme which possesses extended-spectrum properties, followed by bla_{GES-1} and its homologous to bla_{IBC-1}, bla_{GES-2} and bla_{IBC-2} gene cassettes which are found in their sequences [35]. The class 3 integron platform also seems to have been captured by a Tn*402* transposon, yet in the reverse orientation to the class 1 capture event [36]. Class 3 integrons have been described in *Pseudomonas aeruginosa*, *Serratia marcescens*, *Alcaligenes xylosoxidans*, *Pseudomonas putida*, and *Klebsiella pneumonia* isolates from Japan and are not normally recovered in other parts of the world [37].

Class 4 Integron (formerly *intI9*) is embedded in a part of the integrative and conjugative element SXT found on *Vibrio cholerae* chromosome and is known to be an integral component of many γ-proteobacterial genomes. This kind of integron is known as super integron (SI), while integron classes 1–3 are named multi-resistant integrons (RIs). Class 4 integron can be distinguished from RIs because of two reasons: first, a huge number of cassettes that are incorporated, which in the case of *V. cholerae*, the cluster of VCR-associated ORFs presents more than 216 unknown genes in an array of 179 cassettes and occupies around 3% of the genome; and second, the high homology among the *attC* sites of those assembled cassettes. This has been characterized among *Vibrionaceae*, *Shewanella*, *Xanthomonas*, *Pseudomonas*, and other proteobacteria [25, 38]. Class 5 Integron is located in a compound transposon carried on the pRSV1 plasmid of *Alivibrio salmonicida* (GenBank AJ277063) [25].

EPIDEMIOLOGY

Despite the spread of antibiotic resistance which is caused by most of the different types of integrons, there were a few reports about them until 1999 due to the limited scope of knowledge. In 1999, Clark et al identified class 1 integron-related gene, *aadA*, in E. faecalis strain W4470 [39]. Class 1 integron has been specified as a significant type of integron which is connected with different resistance gene cassettes. Class 1 integron, encoding sulfonamide resistance "*sul1*", was inserted into the Tn*402* class 1 integron, removing the end of the *qacE* gene and its attendant *attC* [40], generating the 3' conserved segment (3' CS). Different additional deletions to the Tn*402* element resulted in generation of diversity in the 3' end of the Tn402 class 1 integrons [41]. The class 1 integron integrase recruits gene cassettes from other classes of integron and causes antibiotic resistance, which is affected by selective pressure to generate recent cassette arrays. Therefore, the Tn*402* class 1 integrons has obtained gene cassettes which caused various antibiotic classes [42]. The clinical significance of class 1 integrons has been documented for different bacterial species especially in a large number of gram-negative bacteria, occurring in humans, in various animal species, and even in the environment. Human waste streams of clinical class 1 integrons are progressively being reported as "pollutants" of natural environments [29]. Several studies have shown that class 1 integron can be presented at

multiple strains, and then becomes prevalent by a single, multiple, or combination of integrons via conjugation and natural transformation [43]. Several studies presented the existence of class 1 integrons in *E. coli O157:H7* a, shiga toxin-producing *E. coli* serotypes, and *Salmonella enterica* serovar Typhimurium DT104. Outbreaks of V. cholerae infection have presented the existence of class 1 integrons; therefore, some multi-resistant strains are located on integrons. One sample was *V. cholerae* O1 isolates from Vietnam. Prior to 1991, nobody could find integron, yet after that time, a new ribotype emerged which contained a class 1 integron with an *ant*(3'')- *1a* gene cassette [44]. According to a recent microarray-based comparative genomic analysis of several *V. cholerae* isolates, the O1 and O139 isolates contained more than 95% of the integron cassettes in the superintegron of the O1 serotype N16961, whereas non-O1, non-O139 isolates contained just 50–75% of these cassettes [45]. An interesting point is that presenting class 1 integrons in isolates were acquired from cattle, swine, chickens, fish, and pets such as dogs and zoo animals [46]. Therefore, isolates carrying integrons are not limited to bacteria pathogens. Moreover, there is a steady connection between resistance in humans and in farm animals. Thus, in *Salmonella enterica* serovar Typhimurium TD104, which is endemic in several countries and is highly virulent in humans, two class 1 integron cassettes have been recognized, responsible for resistance to multiple antibiotics. Nesvera (1998) could detect a 29-kb plasmid pCG4 which is associated with streptomycin/spectinomycin resistance determinant from *Corynebacterium glutamicum* in the first evidence of gram- positive bacteria [47]. Tauch et al (2002) could illustrate an *intI1*-like gene on a 27.8-kb R-plasmid pTET3 from C. *glutamicum* LP-6, encoding streptomycin, spectinomycin, and tetracycline resistance; likewise, within pTET3, an aminoglycoside adenyltransferase gene cassette *aad*A9 was detected. Nandi et al (2004) showed class 1 integron in gram-positive organisms like *Corynebacterium sp*. consisting of *C. ammoniagenes, C. casei and C. glutamicum), Aerococcus sp., Brevibacterium thiogenitalis*, and *Staphylococcus sp.* (including *S. lentus, S. nepalensis and S. xylosus*) [31, 43]. In-vitro transfer frequencies of 10^{-2} to Escherichia coli for class 1 integrons were observed, while a comparable result for class 2 integrons was previously reported [48]. Most of the reports about the presence of integrons come from Western Europe and Eastern Asia and also Africa and South America [49]. The first report of class 1 integron in *E. faecium* and *E.faecalis* which contained three different types of cassette arrangement (dfrA12-orfF-aadA2, dfrA17-aadA5, and aadA2) were illustrated by Xu et al (2010). In their continuous studies during 2001 to 2006, Xu et al found that 122 out of 262 Staphylococcus strains were positive for class 1 integrase (*intI*1), 115 (94.3%, 115/122) strains contained the typical 3'CS of *qacEΔ*1-*sul*1 and 4 different kinds of gene cassettes: *aadA2, dfrA12-orfF-aadA2, dfrA17-aadA5,* and *aacA4-cmlA1*. The most isolated resistance genes between *Staphylococcus* strains were *aad*A (95.9%, 117/122) and *dfr*A (52.5%, 64/122) and the rate of integron identification was 60.6% (20/33) in 2001, 60.0% (24/40) in 2002, 50.0% (12/24) in 2003, 45.5% (20/44) in 2004, 42.5% (34/80) in 2005, and 30.0% (12/40) in 2006 [50]. Class 1 integron can interfere with natural selection, especially when applied to organisms with large population sizes, rapid generation times, and access to genetic novelty new gene cassettes encoding antibiotic resistance and other adaptive phenotypes [51].

Class 2 integrons are seen in some isolates such as *Acinetobacter* isolates from Chile, *Enterobacteriaceae* from the urinary tract, *Escherichia. coli*, and *Burkholderia cepacia* [52]. Despite limited knowledge on the distribution of class 2 integrons in the bacterial population, the studies of epidemiology have demonstrated a high-frequency class 2 integron in *Helicobacter pylori* isolates from Argentina [53], *Shigella sonnei* isolates from Australia [54] and, at a lower frequency, different serovars of *Salmonella spp*. from Japan, Argentina, and Spain [55]. Xu *et al*, through their study from 2005 to 2009 recognized 3 out of 16 multidrug-resistant strains of known or opportunistic gram-negative bacterial pathogens to be positive of class 2 integron from the Salmon River in south-central British Columbia [56]. According to the BfT-GermVet monitoring study in Germany during 2004-2006, out of 417 *E. coli* strains, 31 (7.4%, 31/417) were

positive for class 2 integron. Moreover, 3 discovered cassettes are *dfrA1-sat2-aadA1* (77.4%, 24/31), *estX-sat2-aadA1* (19.4%, 6/31), and *estX-sat2-ΔaadA1* (3.2%, 1/31) [57]. The other variants of the class 2 integron integrase (*intI*2) have been described onto an *IncP* plasmid in uropathogenic *Escherichia coli*, which were found to be in association with Tn7. This plasmid includes a glutamine codon (CAA) which removes the stop codon, and is capable of recombination reactions, with its cassette array carrying a gene for trimethoprim resistance. In addition, it carries an unusual cassette, a lipoprotein signal peptidase which is associated with pathogenicity.

Although class 3 integrons were originated from environmental bacteria, they were described, for the first time, from clinical contexts in Japan. [58]. Two species of *Delftia* contain typical chromosomal integrons with class 3 integrases with unknown functions [59]. They are partly common in some human pathogens and commensals in Japan, but not in other parts of the world [35, 60], because the class 3 integron integrase is less active than those of the other ones [60-65].

In conclusion, it is well established that the dissemination of antibiotic resistance genes among bacterial isolates is mediated by integrons. Therefore, in order to recognize mechanisms which facilitate the acquisition of integron by bacteria, more studies over the epidemiology of integron mediated antibiotic resistance is needed.

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