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Resistance integrons and super-integrons

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ABSTRACT

Integrons are genetic elements composed of a gene encoding an integrase, gene cassettes and an integration site for the gene cassettes (*att*). The integrase excises and integrates the gene cassettes from and into the integron, but integrons themselves are not mobile. Two groups of integrons are known: resistance integrons and super-integrons. Nearly all known gene cassettes from resistance integrons encode resistance to antibiotics or disinfectants. These integrons are found on transposons, plasmids and the bacterial chromosome. Gene cassettes in super-integrons encode a variety of different functions. Super-integrons are located on the bacterial chromosome. More than 100 gene cassettes may be present, in contrast to resistance integrons where less than ten cassettes are present. Many species harbour super-integrons, which are species-specific, whereas particular resistance integrons can be found in a variety of species. The gene cassettes in resistance integrons probably originated from super-integrons. In the last few years, a variety of new gene cassettes have been described. Many of these encode resistance against newer antibiotics such as cephalosporins and carbapenems. Resistance integrons have been found in isolates from a wide variety of sources, including food.

Keywords Antibiotic resistance, gene cassettes, integrons, resistance, super-integrons

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INTRODUCTION

Integrons were defined by Hall and Collis [1] as elements which contain the genetic determinants of the components of a site-specific recombination system that recognises and captures mobile gene cassettes. An integron includes the gene for an integrase (*int*) and for an adjacent recombination site (*attI*). Gene cassettes are not necessarily part of the integron, but they become part of the integron when integrated.

Integrons can now be divided into two major groups: the resistance integrons (RI) and the super-integrons (SI). RI carry mostly gene cassettes that encode resistance against antibiotics and disinfectants, and can be located either on the chromosome or on plasmids. The large chromosomally-located integrons, which contain gene cassettes with a variety of functions, belong to the SI group.

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Three classes of RI are known [1–5]. SI are not given a specific name. The integron originally designated as class 4 is now named *Vibrio cholerae* SI. Most RI belong to class 1 (Fig. 1), and class 1 integrons have been reported in many Gram-negative genera including *Acinetobacter* [6–10], *Aeromonas* [11–13], *Alcaligenes* [14], *Burkholderia* [15], *Campylobacter* [16], *Citrobacter* [17], *Enterobacter* [18,19], *Escherichia* [18,20–28], *Klebsiella* [29], *Pseudomonas* [13,30–33], *Salmonella* [34–53], *Serratia* [54], *Shigella* [55–57] and *Vibrio* [58–61]. They have also been found in other bacteria such as *Corynebacterium glutamicum* [62] and *Mycobacterium fortuitum* [63], and a gene cassette has been discovered in *Enterococcus faecalis* [64].

Class 2 integrons are embedded in the Tn*7* family of transposons and consist of an integrase gene followed by gene cassettes [3]. Class 2 integrons have been found in *Acinetobacter* [56], *Shigella* [65] and *Salmonella* [66].

Class 3 integrons have been described in *Pseudomonas aeruginosa*, *Serratia marcescens*, *Alcaligenes xylosoxidans*, *Pseudomonas putida* and *Klebsiella pneumoniae* isolates from Japan. The structure of

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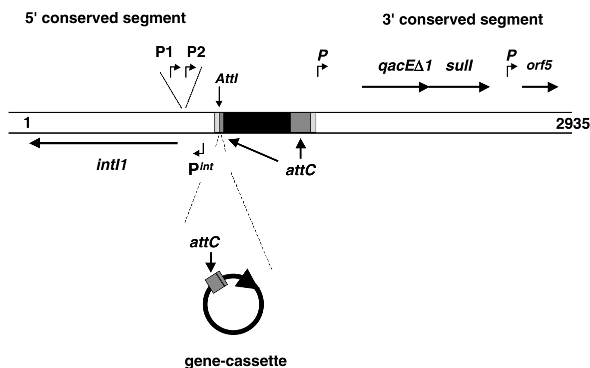

Fig. 1. Schematic representation of a class 1 integron. P1, promoter for trancription of gene cassettes; P2, second promoter that is usually inactive; *int*, integrase gene; *attI1*, integration site; *qacE*, partially deleted gene that encodes quaternary ammonium compound resistance; *sulI*, sulphonamide resistance; *orf5*, unknown function; P, promoters of the *qacE*Δ and *sulI* genes; *attC*, sequence on the gene cassette recognised by the integrase.

class 3 integrons is comparable to that of class 2 integrons [4,5].

SI have been described for *Geobacter sulfurreducens* [67], *Listonella pelagia* [68], *Nitrosomonas europaea* [68], *Pseudomonas alcaligenes* [69], *Pseudomonas mendocina* [69], *Pseudomonas* spp. [69], *Pseudomonas stutzeri* [69], *Shewanella oneidensis* [70], *Shewanella putrefaciens* [67], *Treponema denticola* [67], *Vibrio anguillarum* [71], *Vibrio cholerae* [71,72], *Vibrio fischerii* [73], *Vibrio metschnikovii* [73], *Vibrio mimicus* [71,74], *Vibrio parahaemolyticus* [67,74,75] and *Xanthomonas campestris* [68].

This review describes data published between 1999 and the end of 2002. For data from older literature we refer to appropriate review papers wherever possible.

EPIDEMIOLOGY

Until 1999 only a few reports had described the epidemiology of class 1 integrons, but it was concluded from these reports that integrons were likely to be common around the world, especially in Enterobacteriaceae, and that they contributed to antibiotic resistance [76]. This knowledge has now been supplemented and extended by a large number of further reports. However, knowledge about other classes of integrons is scant.

Class 1 integrons are associated with a variety of resistance gene cassettes, but most integrons contain an *aadA* resistance determinant, encoding streptomycin–spectinomycin resistance. Trimethoprim resistance determinants are also detected frequently. This is not surprising because trimethoprim + sulphamethoxazole has been a therapeutic combination used frequently. Class 1 integrons isolated from bacteria involved in infections of man frequently also harbour gene cassettes encoding β-lactam resistance.

Several groups have reported that integron-containing isolates tend to be more antibiotic-resistant than isolates lacking an integron obtained from comparable patients [13,29,33,59]. Analysis of 867 isolates belonging to eight species obtained from 23 centres in Europe showed that multiresistance is associated significantly with the presence of an integron in these species [77].

Several studies have described the presence of a class 1 integron in multiple strains or species [15], outbreaks caused by a single strain with an integron [17,29,33,59], multiple integrons [78,79], or a combination of these factors [8–11,18,19,21,32,55,58,80]. Remarkably, most of these reports come from Western Europe and Eastern Asia, but there are also reports from Africa [55,60,81] and South America [13,65,81]. In one report, at least nine different integrons were isolated among seven species during a 6-month period from the wards of two specialties [82]. In addition, isolates may contain multiple integrons [61,76,83,84]. These data confirm earlier reports that integrons are ubiquitous among antibiotic-resistant Gram-negative clinical isolates. In addition, some combinations of gene cassettes seem to have a local distribution, whereas others seem to have a widespread distribution [79,80]. Class 1 integrons are located frequently on plasmids that can be transferred by conjugation [80]. In-vitro transfer frequencies of 10^{-2} to *Escherichia coli* for class 1 integrons have been reported [84], and in-vitro transfer rates for class 2 integrons to *E. coli* appear to be comparable [81]. This ease of conjugation, and the fact that class 1 integrons are found on both conjugative plasmids and transposons, explains their widespread distribution.

A large variety of cassette combinations has arisen and new cassettes appear to have become integrated into integrons fairly recently (Table 1). A most remarkable case is the adaptation of a *dfrI* cassette in trimethoprim-resistant isolates of *Campylobacter jejuni*. The cassette had a 90-bp repeat, which had a role in adaptation to the trimethoprim concentration in the medium. The number

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Table 1. Recently described gene cassettes and the corresponding encoded proteins

Gene cassette	Encoded protein	Reference
β-Lactam resistance		
bla_{CEF-1}	Extended-spectrum β-lactamase	156
bla_{GES-1}	Extended-spectrum β-lactamase	157,158
bla_{GES-2}	Extended-spectrum β-lactamase	159
bla_{IBC-1}	Extended-spectrum β-lactamase	160
bla_{IBC-2}	Extended-spectrum β-lactamase	161
bla_{IMP-2}	Carbapenemase	7
bla_{IMP-3}	Carbapenemase	162
bla_{IMP-4}	Carbapenemase	14
bla_{IMP-6}	Carbapenemase	14
bla_{IMP-7}	Carbapenemase	163
bla_{VEB-1}	Extended-spectrum β-lactamase	101,137,157
bla_{VIM-1}	Carbapenemase	164
bla_{VIM-2}	Carbapenemase	165–167
bla_{VIM-3}	Carbapenemase	14,168
oxa-10/aadA1	Extended-spectrum β-lactamase/adenyltransferase	101
oxa-11	Extended-spectrum β-lactamase	169
oxa-13	Extended-spectrum β-lactamase	169
oxa-15	Extended-spectrum β-lactamase	169
oxa-16	Extended-spectrum β-lactamase	169
oxa-17	Extended-spectrum β-lactamase	169
oxa-18	Extended-spectrum β-lactamase	169
oxa-19	Extended-spectrum β-lactamase	169
oxa-28	Extended-spectrum β-lactamase	169
oxa-31	Extended-spectrum β-lactamase	170
oxa-32	Extended-spectrum β-lactamase	171
Aminoglycoside resistance		
aadA4	Adenyltranseferase	172,173
aadA5	Adenyltranseferase	174,175
aadA6	Adenyltranseferase	170,176
aadA8	Adenyltranseferase	83
aadA10	Adenyltranseferase	102
aac(3)-Ib/aac(6′)-Ib	Acetyltransferase	103
aac29a	Acetyltransferase	166
aac29b	Acetyltransferase	166
aacA1b/orfG	Acetyltransferase	23,101
ant(3″)-Ii-aac(6′)-IId	Adenyltransferase/ acetyltransferase	54
aphA15	Phosphotransferase	177
Chloramphenicol resistance		
cmlA4		158
cmlA5	Efflux pump	101
Rifampicin resistance		
arr-2	ADP-ribosylation	101,156
Trimethoprim resistance		
dfr13	Dihydrofolate reductase	172
dfr17	Dihydrofolate reductase	173
dfrA17	Dihydrofolate reductase	175
Quaternary ammonium compound resistance		
qacF′	Efflux pump	167
qacI	Efflux pump	101
Quinolone resistance		
qnr	Unknown	106
Other		
Group II intron	Maturase–reverse transcriptase	54
Open reading frames		
orfO[a]	Unknown	54
orfX	Unknown	54,83,139
orfX′	Unknown	54,83,139
orf i′	Unknown	167
orf ii′[b]	Unknown	167
orf iii′[b]	Unknown	167

[a]Weak homology with *qacE* multidrug transporter; [b]gene cassette lacking some typical structures such as the 5′-consensus core sequence.

of repeats was linked directly to the susceptibility of the cells to trimethoprim. The higher the trimethoprim concentration in the medium, the lower the number of repeats. The mechanism behind this repeat instability is probably slipped-strand mispairing. However, the advantage of the repeats under conditions of reduced or absent drug challenge remained unexplained [16].

The source of integrons in the hospital setting is unknown. It has been shown [85] that the community is a potential reservoir, since 20% of 54 newly admitted patients had an integron-carrying isolate. Antibiotic use at farms has long been considered a significant factor contributing to antibiotic resistance, although this is usually denied by the agricultural industry. Nevertheless, several studies have identified class 1 integrons in isolates obtained from cattle [27], swine [22,25,46], chickens [20] and fish [11,86]. In addition, class 1 integrons have been described in isolates obtained from pets such as dogs [22,28] and zoo animals [22]. Integrons have even been detected in a *Pseudomonas* spp. from an apple orchard [30]. Isolates carrying integrons are not limited to pathogens, but have also been found in bacteria from environmental samples, including water samples from fish farms [6,12], an estuarine environment [87] or other environmental sources [46].

The link between antibiotic resistance and (especially) class 1 integrons in bacteria at farms and in man is corroborated further by the observation that class 1 integrons were present in enterohaemorrhagic *E. coli* from cattle, beef and man. However, only two cattle samples and one beef sample contained integron-bearing isolates [27]. Similarly, another study showed the presence of class 1 integrons in *E. coli* O157:H7 and other shiga toxin-producing *E. coli* serotypes [26]. Further evidence is obtained from salmonellae, which are a recognised cause of zoonoses transmitted via food. *Salmonella enterica* serovar Typhimurium DT104 is pandemic, and although apparently of clonal origin, comes in four types with respect to class 1 integrons: those with an integron harbouring an *aadA* gene cassette; the bla_{PSE-1} gene cassette; those with both cassettes; and those that lack integrons [34–36,38,39,41,43,45,46,50,53]. The integrons are located chromosomally, but can be transferred by transduction [86]. The integron-carrying isolates are usually multiresistant, whereas the integron-lacking isolates are more susceptible to antibiotics. Integron-carrying isolates were obtained from cattle, poultry, companion animals, horses, ground meat from various sources, as well as man, and a link between these reservoirs is obvious [34–36,38,39,41,43,45,46,50,53]. Other

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phage types of this serovar also carry integrons, but these differ from those found in phage type DT104, and are present usually on conjugative plasmids. Among these integrons, *aadA* gene cassettes are common [39,40,46,88]. Similar links between class 1 integrons and other serovars of *Salm. enterica* have also been found, including Agona, Chomedey, Djugu, Enteritidis, Goldcoast, Ohio, Panama, Saintpaul, Virchow and (4,5,12:i:) [37,42,44,45,48,49,51]. Nevertheless, despite the evidence that integrons are present in farm animals, a contribution from the human reservoir to the farm reservoir cannot be excluded.

Several outbreaks of *V. cholerae* infection have shown the presence of class 1 integrons. These strains are often multiresistant, but as in other cases, not all resistance determinants are located on the integron [59,89]. *V. cholerae* O1 isolates from Vietnam collected before 1991 lack integrons, whereas after 1991 a new ribotype emerged that contained a class 1 integron with an *ant(3″)*-1a gene cassette [58]. Further research using isolates collected from Thailand showed that other O serotypes also contained class 1 integrons, and at least five different gene cassettes were found. Some isolates even contained two integrons. It should be noted that one of the isolates with two integrons came from seafood and had the same integrons as three strains obtained from a children's hospital in Thailand [61]. These data lend further support to a close link between food production and problems with resistant isolates in the hospital setting. However, the integrons in *V. cholerae* were located on a plasmid in only one report [59].

Far less is known about the epidemiology of class 2 integrons. This class has been reported in *Acinetobacter* isolates from throughout the world [10,81,90–92]. Class 2 integrons frequently have *aadA1*, *dfrA1* and *sat* gene cassettes integrated [78,90–92]. Class 1 integrons may be present in *Acinetobacter* isolates harbouring a class 2 integron [92], and this phenomenon has also been described for *Shigella sonnei* [56]. Usually class 1 and class 2 integrons are found in separate isolates [10,81,88]. Class 2 integrons have also been described in Enterobacteriaceae from urinary tract infections [78].

Until now, no evidence has been obtained to show that SI are exchanged between different strains or bacterial species.

ORIGIN

Much has been speculated about the origin of integrons and gene cassettes. Little is known for certain, although the genetic background and the gene cassette contents of class 1 integrons allow speculation about their evolution. Bissonette and Roy [93] proposed an evolutionary scheme based on In0, a class 1 integron lacking gene cassettes. For example, In2-, In4- and In6-like integrons seem to constitute distinct groups with independent origins [68,94–96].

The sequences of the *attC* sites from gene cassettes in RI and SI suggest that gene cassettes in RI were derived from SI [68]. First, the sequences of the repeats of gene cassettes within an SI seem to be species-specific. Second, the sequences of *attC* sites in RI (equivalent to the repeats of SI) are highly diverse. Third, sequences from some RI gene cassettes are identical to those observed in SI of different species, such as *V. cholerae* [74], *Shew. putrefaciens* or *Xanthomonas* spp. [97], or *P. alcaligenes* [69]. Fourth, *V. cholerae* SI gene cassettes are recognised by IntI1 [57].

This hypothesis was tested further by Rowe-Magnus *et al*. [97]. A plasmid carrying the *intI1* gene, and a plasmid carrying the first two gene cassettes of In3 and the promoter for the gene cassettes, were introduced into a *V. cholerae* strain which was phenotypically antibiotic-susceptible, but which carried gene cassettes in its SI that potentially encoded antibiotic resistance, including resistance to chloramphenicol. After induction of the integrase, thereby allowing transfer of gene cassettes, the In3-bearing plasmid was transferred to *E. coli* and the transconjugants were subjected to antibiotic selection. Analysis of the transconjugants showed that a chloramphenicol acetyltransferase homologue was integrated in *attI1*, demonstrating that gene cassettes from the *V. cholerae* SI could be recruited to a class 1 integron. Analysis of other transconjugants revealed that other gene cassettes had also been recruited. Apparently gene cassettes are integrated at random and useful new combinations arise, which can then be selected by environmental conditions [97]. So at least part of the RI appears to have evolved through the recruitment of SI gene cassettes.

The origin of gene cassettes is unclear. An *attC* primer–reverse transcriptase model for the

genesis of gene cassettes has been proposed [98], but not all researchers agree with this model. In particular, the fact that some gene cassettes are inserted in the opposite direction (generally the coding sequences of gene cassettes are in the correct orientation for transcription from the P_{ANT} in the 5′-CS) or have their own promoter, do not fit well with this model [76,99]. The relatedness of gene cassette repeats within species and genera suggests that the genesis of gene cassettes is linked to the species harboring the SI [76,78]. Nevertheless, SI appear to be very old structures. The divergence of the sequences of integrase genes of SI from different species more or less mirrors the divergence among 16S sequences from these species, thereby indicating that the integrase gene was already present when these species diverged [68,69,100]. Thus, the history of gene cassettes is shrouded in mystery, and the history of the RI integrases is also unclear, as is their association with the conserved segments.

GENE CASSETTES

Gene cassettes consist of a coding sequence, which usually lacks a promoter, followed by a repeat sequence. In RI the repeat sequence is called a 59-base element (59-be) or *attC*. The repeat sequences of SI are named after the species in which the SI resides, e.g., the repeat of a *V. cholerae* gene cassette is called VCR. The repeats of SI are generally species-specific, but some direct or indirect exchange of repeats between some species has occurred [68,69,71,72].

Several new antibiotic resistance cassettes in RI have been described in recent years (Table 1). For an overview of gene cassettes described previously see Fluit and Schmitz [76]. Remarkably, until 1999, nearly all the known gene cassettes encoded resistance to the oldest groups of antibiotics, but an increasing number of new gene cassettes defining resistance against newer groups of antibiotics have now appeared. Even so, some of these antibiotic classes have been used for more than 20 years. RI, like In53, may contain as many as eight gene cassettes. One gene cassette in In 53 was composed of a fusion between two previously known gene cassettes (*oxa-10* and *aadA1*) encoding a β-lactamase and an adenylyltransferase, respectively. In addition, two other new cassettes (*cmlA5* and *qacI*) were found. All three newly discovered cassettes had their own promoter sequences, a feature in contrast to most other known cassettes [101].

It should also be noted that a large proportion of the RI contain gene cassettes encoding resistance against streptomycin and spectinomycin, despite the fact that use of these antibiotics (at least in a clinical setting) has long ago been discontinued. In fact, new gene cassettes encoding resistance against these aminoglycosides have been discovered during the last few years [83,102]. In the case of *aadA8*, the gene cassettes may have arisen following recombination between the *aadA2* and *aadA3* cassettes. So, although new gene cassettes seem at first sight to be rather distantly related, this relationship may be much closer upon better scrutiny.

Sometimes minor sequence differences between gene cassettes have been found, which may result in larger differences at the protein level. For example, the *ereA2* and *ereA1* genes are nearly identical, but a one-nucleotide deletion in the *ereA1* sequence results in a predicted protein that is 62 amino-acids shorter than its EreA2 counterpart. Whether these differences are real or reflect sequence errors is a matter of debate [83].

However, larger deletions may sometimes occur and new fusion cassettes arise, which may yield a functional fusion protein. An example is the product of the fused *aac(3)-Ib* and *aac(6′)-Ib* genes. Not only is the fusion protein functional, but so also are the separately cloned products of the genes, despite the lack of the last four amino-acids in the AAC(3)-I enzyme. This enzyme confers resistance to gentamicin and fortimicin. The AAC(6′) enzyme showed a Leu119Ser amino-acid change when compared to the most closely related enzyme. This mutation slightly affected resistance to amikacin and netilmicin, but not resistance to tobramycin, kanamycin and dibekacin. The fusion product had the combined effect of both proteins, but resistance to fortimicin was lost and resistance to gentamicin was enhanced [103].

Not all class 1 integrons adhere to the standard model (Fig. 1). Sometimes additional (resistance) genes are integrated. Verdet *et al*. [104] described the insertion of a β-lactamase-encoding gene, bla_{DHA-1} (an *ampC* gene) and its regulator gene *ampR*, into an integon. This integron is present on plasmid pSal-1, isolated from a strain of *Salm. enterica* serovar Enteritidis. The genes are inserted behind an integron carrying the *aadA2* and *orf4*

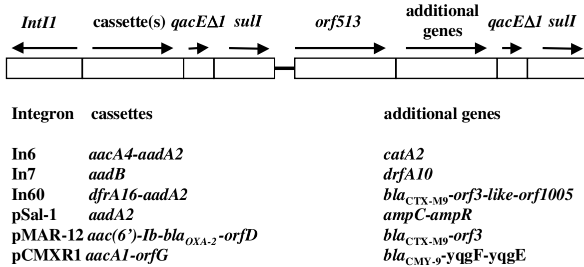

Fig. 2. Schematic presentation of In6-like class 1 integrons, their gene cassettes and additional genes. The direction of transcription for *orf1005, ampC, yqgF*, and *yqgE* is opposite to those for the gene cassettes, as indicated by the arrow. In pSAL-1, *orf341* is present instead of the closely related *orf513*. These genes differ only by the insertion of a G at position 960 of *orf341* to yield *orf513* [94–96,104,140].

gene cassettes. The 3′-CS of this integron consists of the *sulI* and *orf341* genes. Then follow the genes encoding the β-lactamase and its regulator. These genes are then followed by at least part of the 3′-CS (Fig. 2). The AmpC β-lactamase gene and its regulator are almost certainly derived from *Morganella morganii*. This represented the first time an AmpC β-lactamase was recognised in an integron, although the inserted genes cannot be considered gene cassettes because of the absence of *attC*. Furthermore the genes are in opposite orientation. The mechanism of insertion is unknown, but it has been suggested that *orf341* encodes a recombinase [105] that recognises the insertion site of the β-lactamase gene and its regulator.

Another unusual RI was described by Centron and Roy [54]. Isolated from a strain of *S. marcescens*, it contains a group II intron sequence not normally present in bacteria. The first gene cassette is *ant(2″)*-Ia, but the *attC* is separated by a 1971-bp insert encoding the group II intron. The intron encodes a putative maturase–reverse transcriptase. Transcription of the gene is opposite to that of gene cassettes. The *attC* of the first cassette is followed by a gene cassette, *ant(3″)*-Ii-aac(6′)-*IId*, encoding a bifunctional aminoglycoside resistance mechanism. The presence of the group II intron in the integron suggests a role in cassette formation and other aspects of plasmid evolution.

Only two examples of plasmid-mediated quinolone resistance are known, of which one is defined by a gene cassette located on a class 1 integron. The product of this gene is 218 amino-acids in length and belongs to the pentapeptide repeat family of proteins. It has pentapeptide repeat domains of 11 and 28 tandem copies, separated by single copies of glycine. The protein protects DNA gyrase, but not topoisomerase IV, from ciprofloxacin action; however, the precise mechanism is still unknown [106].

The first SI was discovered in *V. cholerae* and was originally called a class 4 integron [74,98]. The SI of strain El Tor N16961 contains 216 open reading frames (ORFs), most of which lack a promoter and have an unknown function. Among recognisable encoded functions are: chloramphenicol acetyltransferase, fosfomycin resistance protein, glutathione transferase, MutT, transposase, integrase, haemagglutinin, lipoproteins, HigA, HigB and Doc (these latter three proteins are similar to host addiction proteins, which are used by plasmids to select for their maintenance by host cells). Thus, these proteins represent a wide repertoire of functions [72]. Some of the ORFs are in opposite orientation when compared with most gene cassettes, which have the normal orientation for gene cassettes in integrons. This picture is consistent with other data for this and other strains of *V. cholerae* [71].

The SI of a *V. metschnikovii* isolate from 1888 was characterised partially and contains 20 unique gene cassettes, of which six are repeated cassettes. Only a few of the gene cassettes seem to have counterparts in *V. cholerae*. Potential gene functions include: restriction enzyme, chaperone, lipolytic enzyme, plasmid partitioning, methyltransferase and glutathione lyase. The repeat sequences (VMR) are related to the VCR of *V. cholerae*, but are not identical. *V. metschnikovii* integrase was called IntI6, but is now known as VmeIntIA [68,73].

SI are not limited to Vibrionaceae, but are also present among pseudomonads. The best-described example is found in *P. alcaligenes*, a non-pathogenic bacterial species. The SI is 18 kb in size, with a total of 33 repeats called PAR (‘*Pseudomonas alcaligenes* repeat’). No ORF was identified for PAR3 and PAR8. Repeats with homology to PAR have also been found in other pseudomonads, but not in species belonging to new genera, such as *Burkholderia* and *Stenotrophomonas* that were formerly included in the genus *Pseudomonas*. None of the ORFs coded for a known protein, although some patterns suggestive of lipoproteins or transmembrane sequences were found [69].

The SI of *Shew. oneidensis* is remarkable among SIs because it contains only three gene cassettes. The first of these encodes a transposase and a recombinase, and forms part of an IS element. The function(s) encoded by the second is (are) unknown. The third consists of a small non-coding sequence. Finally, in the opposite direction there is a copy of the ORF located in the second gene cassette [70].

The ubiquitous nature of SIs was demonstrated further by their presence in randomly taken environmental samples in which three new integrase genes (called *intI6–intI8*) were discovered [67]. In a complementary approach, the presence of gene cassettes in environmental samples was studied, resulting in the discovery of more than 120 gene cassettes. Only a few of the functions encoded by these gene cassettes could be identified, including an aminoglycoside phosphotransferase, a toxin part of a plasmid maintenance system, RNA methyl transferase and pyrimidine dimer DNA glycolase [107].

The question of the stability of the order of gene cassettes is not yet solved. Analysis of predominant integron types in ten European hospitals suggested that these are stable structures [108], but in-vitro studies have achieved changes in gene cassette order readily [109]. The excision of gene cassettes can be mediated by the integrase, and each cassette forms a circular intermediate. Subsequent reintegration of the gene cassettes at the favoured *attI* site could lead to a rearrangement [109]. Apparently this rearrangement occurs only under specific environmental conditions. The gene cassettes within an SI can also change order, as evidenced by the order of gene cassettes in the SIs of two *V. cholerae* strains [71–73,100].

The exchange of cassettes between different integrons has hardly been studied. One study used *Salm. enterica* serovar Typhimurium DT104 as a donor and several plasmids as acceptors. Translocation of gene cassettes was dependent on the acceptor plasmid used. Dependent on the antibiotic selection used, gene cassettes, other determinants or both were translocated. Remarkably, the translocation of larger sections of the *Salmonella* genomic island 1 (SGI1; see below) required the presence of an integron. Probably the integron in the target is required for the recombination associated with the translocation. Thus, this study indicated that the presence of an integron may lead to a more extensive exchange of resistance determinants than gene cassettes alone [110].

EXPRESSION OF GENE CASSETTES

Expression of gene cassettes in class 1 integrons is not uniform. Most gene cassettes in class 1 integrons are expressed from a common promoter located in the 5′-CS region. This region contains two potential promoter sites, P1 (also called P_{ANT}) and P2; however, the latter promoter is frequently inactive because only 14 nucleotides are present between the −35 and −10 boxes of this promoter instead of the optimal 17 nucleotides. At least five different P1 and two different P2 promoters have been described, which may come in varying combinations [2,83,111,112]. These promoters vary in strength [2,112]. P1 is located *c.*200 bp upstream of the integration site.

The position (i.e., order) of a cassette in the integron is related to the level of resistance observed [113]. In fact, the imperfect inverted repeats of *attC* may form stem loop structures, which may silence downstream genes completely [113]. Translation may also be controlled by additional signals, such as a translational attenuation signal, which is (e.g.) present in a cassette encoding inducible chloramphenicol resistance, although these are an exception [114,115]. For many gene cassettes, the translation initiation region (TIR; comprising initiation codon, the Shine-Dalgarno sequence and an adequate spacer) appears to be present. Many of the coding regions of these cassettes are located 2–62 nucleotides downstream from an ORF with a coding capacity for 11 amino-acids. This ORF is separated by eight nucleotides from a consensus Shine-Dalgarno sequence. This region is located close to the *attI1* site, and the stop codon of the ORF is located in the 7-bp core site (GTTRRRY) [116]. This ORF is named ORF-11, and is created by recombination when the core sites contain a stop codon [117]. Thus, ORF-11 spans *attI1 and* supports the expression of *aacC1* of Tn*1696*. ORF-11 is fused to the ORF of this cassette through a 19-bp duplication of a sequence near the *attI1* site [118]. Similar mechanisms are present in a few other genes inserted at the *attI1* site [117]. However, ORF-11 by itself, and especially the TIR, appears to play an important role in the expression of some gene cassettes. The translational efficiency for the *aac(6′)-Ib7* gene dropped dramatically (by

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80%) when both TIR and ORF-11 were deleted. Invalidation of the ATG start codon of ORF-11 or its putative Shine-Dalgarno sequence resulted in a 60% drop in translation. However, mutagenesis of ORF-11 or a change in distance to the *aac(6')-Ib7* sequence had a less dramatic effect. So, the translation of TIR-deficient gene cassettes may benefit substantially from the presence of the TIR of ORF-11 [117].

INTEGRASE

The integrases of integrons belonging to classes 1–3 are named IntI1–3, but the naming of integrases after IntI4 is confusing. The designations IntI5 and IntI6 were used for different integrases [67,68,71,73]. The naming system used by Rowe-Magnus circumvents this problem. IntI4 is now called VchIntIA, whereas, for example, the integrase of *V. metschnikovii* is called VmeIntIA [68].

Integron integrases belong to the tyrosine recombination family. This family of enzymes is characterised by the presence of invariant RHRY amino-acids in the conserved motifs called box 1 and box 2. In addition, three other motifs, possibly involved in secondary structure, were identified and called patches I–III [119], while a lysine residue is of catalytic importance [120,121]. Further study of the alignments of amino-acid sequences of integron integrases with the amino-acid sequences of other family members showed the presence of an additional domain in the first group. This domain is found approximately two-thirds of the way along the sequence of the protein and is about 35–40 amino-acids long. Mutation analysis suggested that part of the domain is involved in DNA binding, whereas another part is involved in recombination activity [121].

The class 1 IntI1 integrase recognises three types of recombination site: *attI1*, *attC* and secondary sites. Binding domains and consensus sequences have been determined for these. The *attI1* site is a simple site which consists of two inverted sequences that bind the integrase, and two additional integrase-binding sites called strong and weak (also known as DR1 and DR2, respectively) (Fig. 3) [122–124]. The *attC* region consists of four essential sites called 1R, 2R, 1L and 2L. The 1R and 2R sites are part of the RH consensus sequence, which is more or less equivalent to the RH simple site. The 1L and 2L sites

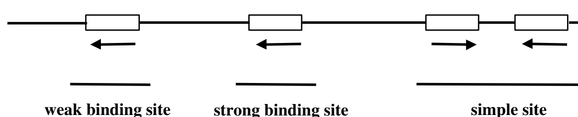

Fig. 3. Schematic presentation of the *attI1* site. The essential part of the *attI1* site is formed by the simple site, the strong binding site and the intervening sequence. For details see Hall *et al.* [123].

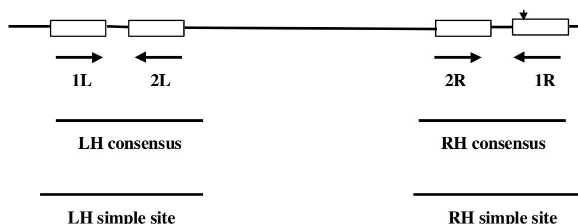

Fig. 4. Schematic presentation of an *attC* site. The vertical arrow indicates the recombination point. The core site sequences of the simple sites are boxed. For details see text and Hall *et al.* [123].

are part of the LH consensus sequence, which is more or less equivalent to the LH simple site (Fig. 4) [122,123]. For the secondary sites, a consensus sequence Ga/tT has been defined [123]. The integrase binds only to the single-stranded DNA of the bottom (3'–5') strand of *attC*. This binding capacity is confined to the carboxyl-terminal of the protein. However, the integrase could bind both double-stranded and bottom strand single-stranded *attI1* DNA, but the carboxy-terminal part of the integrase was not able to bind *attC*. Footprinting of the *aadA1 attC* showed that 40 nucleotides were protected, including two of four proposed integrase binding sites [122]. The LH and RH sites in the *attC* are possibly distinguished by the integrase, which might explain the orientation of integration of the gene cassettes. L2 also appears to be important for orientation. The LH simple site is not only required for orientation, but also enhances RH activity [123].

Recombination between an *attI1* site and an *attC* is favoured slightly over recombination between two *attC* sites, but a recombination between two *attI1* sites is far less efficient. Also, recombination of an *attI1* site with a secondary site is less efficient than recombination of *attC* with a secondary site [125,126]. The full (65-bp) *attI1* site is required for efficient recombination with *attC* [126], although some authors suggest that a smaller sequence is essential, with

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contributions from flanking sequences [123]. The differences between these views may be explained largely by the definition of efficient recombination. However, the presence of only the simple site is sufficient to obtain a low frequency of recombination. Nevertheless, the *intI1* integrase uses secondary sites for integration of a gene cassette. This integration may yield a secondary site and a hybrid *attC*. This hybrid 59-be appears to be fully recombination-proficient. However, this sequence is probably insufficient to allow excision of the gene cassette [124].

During recombination, DNA strands are cut, but the mechanism is not elucidated completely. Some authors have suggested that only one strand is cut by the integrase, followed by resolution of the resulting Holliday junction by a cellular enzyme such as RuvC [127]. However, experiments in a *ruvC* mutant did not show impaired recombination, and it has been hypothesised that the integrase cleaves both strands in a staggered fashion. This mechanism would be similar to that used by XerC–XerD recombinases [128].

Recombination events do not always occur as predicted. Secondary insertion sites are an example, but unusual recombination between an *attI1* site and an *attC* may be responsible for the observed loss of the central part of the *attC*, thereby creating a potential fusion of two adjacent gene cassettes [126]. Another potential example is the *aadA10* gene cassette in plasmid R151. This gene cassette has only the LH simple site, and integrase-mediated excision could not be detected [102].

The class 2 integrase IntI2 is, in all cases so far sequenced, not functional because of the presence of an internal stop codon. The amino-acid sequences of IntI1 and IntI2 are < 50% homologous. Replacement of the internal stop codon with a codon for glutamic acid yields a functional integrase. Although gene cassettes found in class 2 integrons are identical to those in class 1 integrons, the functional IntI2 was not able to excise gene cassettes from class 1 integrons. In contrast, IntI2 was able to both excise and insert gene cassettes into class 2 integrons [129]. The reason for the stop codon is unclear, but the *intI2* gene may be a pseudogene. The integration and excision of gene cassettes is then dependent on the presence of another type of integrase. A prime candidate is IntI1, encoded by class 1 integrons found frequently in the same isolates as class 2 integrons. The non-functional IntI2 may also explain why only a small number of different gene cassettes have been observed in class 2 integrons when compared to class 1 integrons. Another possible explanation for a truncated IntI2 is a regulatory function. The DNA-binding domain of the integrase is still present, and thus the protein may bind to the same sites as the full protein. However, the advantage of such a function remains unexplained [129].

The properties of the IntI3 integrase from class 3 integrons are similar to those of the IntI1 integrase. IntI3 integrase is able to recognise different 59-be elements and integrate the cassettes into the *attI3* site. Integrated cassettes can also be excised by this integrase. Recombination between *attC* and secondary integration sites is also catalysed by IntI3 integrase, but at lower frequencies than IntI1 integrase [123,130]. The resemblance of class 1 and class 3 integrons is exemplified further by the fact that gene cassettes appear sometimes to cross integron class borders. Bla_{IMP} was first described in a class 3 integron [5,130], but has been isolated subsequently from a class 1 integron [131].

The increasing number of integron classes discovered also leads to the characterisation of new integrases. One example is the IntI-like recombinase of a *Shew. oneidensis* isolate from lake sediment. This integrase is 45% identical to IntI1 and belongs to a small chromosomally-located integron. This integrase excises gene cassettes readily when they are flanked by two *attC* sites, or when the *attI2* site is adjacent at one end. Excision does not take place when the *attI1* or *attI3* sites are present. The integrase is able to integrate gene cassettes in the cloned *attI* site from the *Shew. oneidensis* integron [70].

The *VchIntIA* (*intI4*) genes from different strains are nearly identical [71,74]. Similarly, the *VmiIntIa* (*intI5*) gene sequence, encoding the *V. mimicus* SI integrase is 75% identical to the sequence for *VchIntIA*, whereas non-coding sequences are 65% identical [71].

GENETIC SURROUNDINGS OF INTEGRONS

Integrons are found frequently as part of transposons, including Tn*21*, Tn*1403*, Tn*1404*, Tn*1696*, Tn*1412* and Tn*2000* [25,27,52,57,101,102,132–135].

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These transposons may be located either chromosomally or on a plasmid. The plasmids are often large (> 100 kb) conjugative plasmids belonging to (e.g.) groups IncFI [52,134], IncFII [47], or IncL/M [40].

Class 1 integrons have been associated with a variety of insertion sequence (IS) elements, including IS*26* [90,98] IS*1999* [136–138], IS*2000* [136,137] and IS*6100* [133,139]. Most commonly, IS*6100* has been found at the 3′-end of integrons. These could be either complete integrons such as In4, which has a 3′-CS that includes *qacE*Δ1, *sulI*, *orf5* and *orf6*, or truncated versions of this region that have a complete absence of the 3′-CS [133]. The IS*6100* elements also border Tn*610* of *Mycobacterium smegmatis*. The IS elements in this transposon are bordered by sequences derived from the 5′- and 3′-CS of integrons, respectively, but the integrase and the *attI1* site are partially deleted, resulting in an inactive integron [133].

The IS elements associated with integrons are not always situated outside of the integron. In two integrons, In50 and an integron from *P. aeruginosa*, the IS elements are directly behind the 5′-CS. In the case of In50, both IS*1999* and IS*2000* are present, whereas only IS*1999* is found in the *P. aeruginosa* integron. The transcription of the IS elements is opposite to that of the gene cassettes. Interestingly, both integrons contain a $bla_{\mathrm{VEB\text{-}1}}$ cassette and an *aadB* cassette. The *P. aeruginosa* integron also contains the *arr-2* and *cmlA* gene cassettes [140].

In two cases (*Mycobacterium fortuitum* and *K. pneumoniae* isolates with a class 1 integron), the IS was inserted in the integrase-encoding gene of the class 1 integrons, but for In72 in *P. aeruginosa* the truncation of the integrase gene was not caused by such an insertion [31,63].

In some cases, recombination between two integrons has been identified. The In-t1/In-t2 fusion in Tn*21* on an IncFI plasmid is such an example. The first integron consists of the integrase gene followed by the *aadB* and *catB3* gene cassettes, the *sulI* gene, an extended version of *orf341*, and a *dfrA18* gene. This gene arrangement is then followed by the *oxa-1* and *aadA1* gene cassettes and a *sulI* gene, which form In-t2 [52]. Another example may be formed by the In6 and In7 family of integrons. These integrons share a common backbone pattern, with a notable feature being a duplication of the 3′-CS region (Fig. 2) [94–96,104]. The integron on plasmid pCMXR1 also seems to belong to this family, although only the duplicated 3′-CS was identified [141]. Recombination between a class 1 and a class 2 integron has also been described. The hybrid had the IntI2 integrase of class 2 integrons, but the 3′-CS was derived from a class 1 integron [92].

The integron that contains the $bla_{\mathrm{PSE\text{-}1}}$ gene cassette (sometimes present in *Salm. enterica* serovar Typhimurium DT104), is linked downstream to a copy of IS*6100*. A second integron carrying the *aadA2* gene cassette is sometimes located upstream in these isolates. In between, the *floR* and *tetR/tetA* genes are present, which encode resistance to florfenicol and tetracycline, respectively. The whole region is part of a structure called *Salmonella* genomic island 1 (SGI1) [142,143]. SGI1 is 43 kb in size and has 44 coding regions, most with unknown function, and is bounded by two 18-bp repeats that suggest specific integration. Thus SGI1 is potentially a mobile genetic element [143–145]. SGI1 seems to be a hot-spot for the integration of resistance determinants, and several variants have been described, including in *Salm. enterica* serovar Agona [146].

In some cases, a class 1 integron is associated with a *res* site [133,147,148]. This site can be recognised by the *uvp1* resolvase, which is almost identical to the *resP* resolvase [133,149–151]. The sequences present in some cases indicate that these integrons can move by a mechanism similar to those described for some transposons [134,152]. In fact, two integrons were first described as transposons [134], and some are defective transposons [153]. However, other mechanisms of insertion may play a role at other sites, but this is still either a matter of debate [151,154] or unknown.

Mobile genetic elements are believed generally to carry either virulence determinants or resistance determinants, but exceptions exist. One such example is a 140-kb IncFII plasmid isolated from *Salm. enterica* serotype Typhimurium isolates belonging to four different phage types. This plasmid carried the *spvA-C* (*Salmonella* plasmid virulence) and *rck* (resistance to complement killing) determinants and a class 1 integron with the *oxa-1* and *aadA1* gene cassettes. In addition, sequences characteristic of Tn*21* were present, although a link between this transposon and the integron could not be established. The presence of both resistance and virulence determinants on a single plasmid is a new and interesting example

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of plasmid evolution, but poses new challenges to public health [47].

The SI, by definition, are located on the chromosome. The neighbouring sequences, except for those of *V. cholerae,* are hardly known. The occurrence of a SI in one species of a genus is not necessarily predictive for other species in that genus. This is clearly illustrated by the genus *Vibrio*. The SI from *V. cholerae* is located on the small chromosome [72], whereas mapping of the *V. parahaemolyticus* genome showed that its SI was located on the large chromosome [71,75]. The diversity of genetic backgrounds in which RI are found still requires a satisfactory explanation.

CONCLUDING REMARKS

It can be concluded that the gene cassettes found in SI encode a wide variety of different functions, in contrast to the functions of gene cassettes found in RI. The emergence of new gene cassettes in class 1 integrons, especially those with resistance mechanisms directed against the newer β-lactam antibiotics and fluoroquinolones is a cause for concern. Also, the number of resistance genes carried by the same plasmid, and even in the same integron, appears to rising. However, the integration of virulence factors and resistance determinants on the same plasmid may have even greater implications for public health. These bearers of multiresistance are likely to remain, because the physical association of integrons with other resistance determinants will lead to their continuous selection [77]. Thus, a decrease in sulphonamide prescription in the UK did not lead to a decrease in the number of RI-positive isolates [155]. The role of SI in the evolution of bacterial species has been barely touched upon, but their apparent ubiquity suggests that they play an important role in bacterial evolution. The variety of structures found among class 1 integrons and their genetic surroundings after slightly more than half a century of antibiotic usage bears testament to the genetic flexibility and adaptability of the bacterial genome under environmental stress, making these microorganisms ultimate survivors.

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