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Integrons: Past, Present, and Future

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SUMMARY

Integrons are versatile gene acquisition systems commonly found in bacterial genomes. They are ancient elements that are a hot spot for genomic complexity, generating phenotypic diversity and shaping adaptive responses. In recent times, they have had a major role in the acquisition, expression, and dissemination of antibiotic resistance genes. Assessing the ongoing threats posed by integrons requires an understanding of their origins and evolutionary history. This review examines the functions and activities of integrons before the antibiotic era. It shows how antibiotic use selected particular integrons from among the environmental pool of these elements, such that integrons carrying resistance genes are now present in the majority of Gram-negative pathogens. Finally, it examines the potential consequences of widespread pollution with the novel integrons that have been assembled via the agency of human antibiotic use and speculates on the potential uses of integrons as platforms for biotechnology.

INTRODUCTION

Integrons are genetic elements that allow efficient capture and expression of exogenous genes. They are widely known for their role in the dissemination of antibiotic resistance, particularly among Gram-negative bacterial pathogens. However, since their initial discovery in clinical contexts, it has become apparent that integrons are a common component of bacterial genomes and that they have a long evolutionary history. Integrons occur in all environments, are able to move between species and lineages over evolutionary time frames, and have access to a vast pool of novel genes whose functions are largely yet to be determined. Over the last decade, exploration of integron diversity in natural environments has shown that they are more than just a curious feature of antibiotic-resistant pathogens but have a more general and important role in bacterial adaptation and genome evolution.

This review examines the natural history of integrons. It explores the activities of integrons in the general environment and the mechanisms by which they sample and rearrange their stock of gene cassettes. It shows how clinically relevant integrons arose by sampling genes from diverse environmental sources, speculates on the future evolutionary trajectory of integron systems, and explores the potential use of integrons in biotechnology.

STRUCTURE AND NATURAL HISTORY OF INTEGRONS

Structure of Integrons

All integrons share three essential core features, whose combined activities capture and subsequently express exogenous genes as part of gene cassettes (1, 2). The first feature is *intI*, a gene which encodes an integron integrase (IntI), a member of the tyrosine recombinase family (3). The integron integrase protein catalyzes

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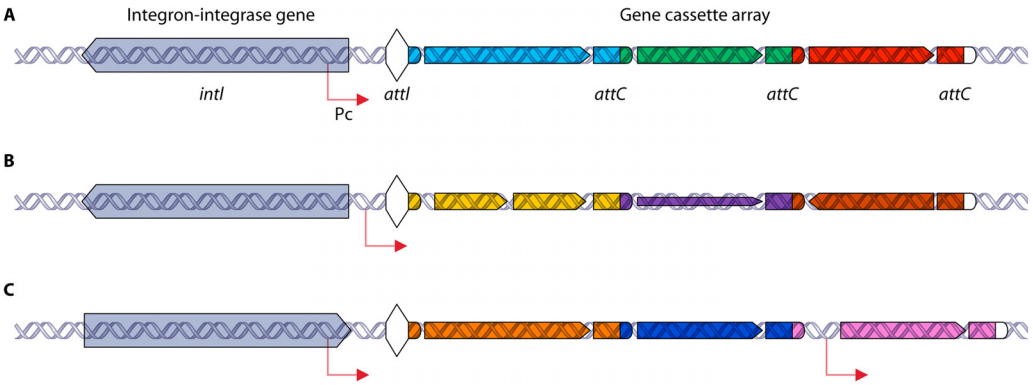

FIG 1 Integron structure. The basic integron platform consists of the following: *intI*, a gene for the integron integrase; Pc, an integron-carried promoter; *attI*, the integron-associated recombination site; and gene cassettes, sequentially inserted into an array via recombination between *attI* and the cassette associated-recombination sites, *attC*. (A) Gene cassettes normally contain a single open reading frame (ORF) (arrow) expressed from the Pc promoter. In some integrons, Pc lies between *intI* and *attI*. (B) Cassettes with two ORFs, no ORF, or an ORF in the reverse direction are known. In some genera, *intI* is transcribed in the same direction as the gene cassettes. (C) Gene cassettes may also contain internal promoters.

recombination between incoming gene cassettes and the second core feature, an integron-associated recombination site, *attI* (4). Once a gene cassette is recombined, it is expressed by the third core feature, an integron-associated promoter, Pc (Fig. 1) (5, 6).

Integrons acquire new genes as part of gene cassettes (7). These are simple structures, usually consisting of a single open reading frame (ORF) bounded by a cassette-associated recombination site, originally called a 59-base element but now referred to as *attC* (8, 9). Circular gene cassettes are integrated by site-specific recombination between *attI* and *attC*, a process mediated by the integron integrase (10) (Fig. 2). This process is reversible, and cassettes can be excised as free circular DNA elements (11–13). Insertion at the *attI* site allows expression of an incoming cassette, driven by the adjacent Pc promoter (5).

The integron system has two key advantages as a means of genomic innovation. First, new genetic material is integrated into the bacterial genome at a specific recombination site (*attI*) and thus does not perturb existing genes. Second, the newly integrated gene is then expressed via the integron promoter (Pc) and therefore is instantly ready to be subjected to natural selection. Consequently, in a population of integron-containing cells, each of which samples different gene cassettes, any newly generated variants will immediately express genes that might confer advantageous phenotypes.

Evolutionary History of Integrons

Integrons are genetic loci defined by the presence of a gene encoding the integron integrase, IntI. The integron integrases are members of the tyrosine recombinase family but are characterized by having an additional unique 16-amino-acid conserved motif necessary for activity (3, 14). On the basis of carrying an IntI gene (*intI*), more than 15% of genome-sequenced bacteria appear to contain an integron (1, 2). Integrons are also found in a wide diversity of environments, including forest soils, desert soils, riverine sediment, Antarctic soils, hot springs, aquatic biofilms, plant surfaces, marine sediment, and deep-sea sediment (15–20).

Different integrons can be distinguished based on the relative homology of *intI*, although a percent cutoff point to discriminate between different "classes" of integrons has not been formally defined. Nevertheless, it is clear that hundreds of different inte-

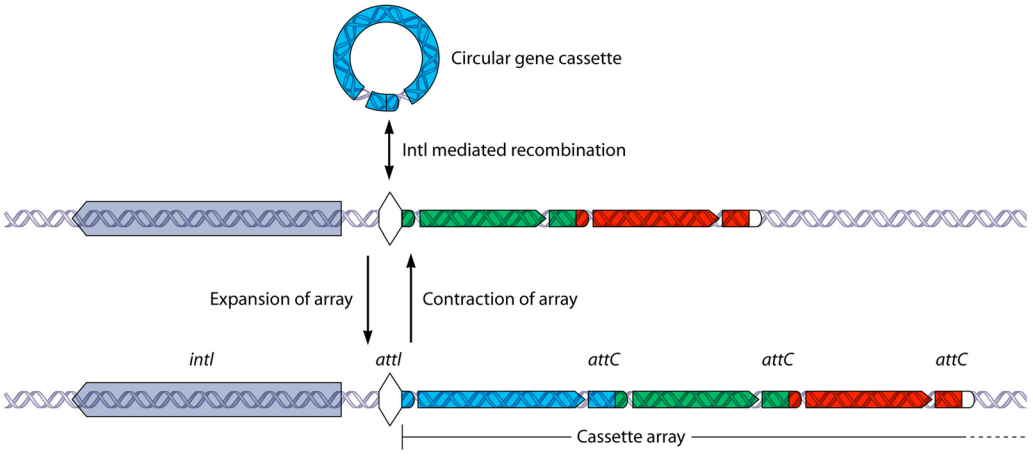

FIG 2 Acquisition of gene cassettes. Integrons acquire new gene cassettes by recombination between the *attC* of a circular cassette and the *attI* site of the integron. This inserts incoming cassettes at a position proximal to the integrase gene and its embedded promoter. Cassette arrays can expand by repeated cassette acquisition, but cassettes can also be excised as closed circles by *attI* × *attC* or *attC* × *attC* recombination.

gron families have been discovered in the last decade (1). These integrons fall into three broad groups based on the phylogeny of their respective integrase genes: (i) a group found in proteobacteria from freshwater and soil environments, which also includes the clinically important class 1 and 3 integrons; (ii) a group found in gammaproteobacteria from marine environments, which includes the class 2 integrons and integrons found on the SXT integrative conjugative element and pRSV1 plasmid from *Vibrio*; and (iii) integrons whose integrase genes are in the reverse orientation to those listed above (Fig. 1). These reverse integrons have so far been found in members of the *Spirochaetes*, *Planctomycetes*, *Cyanobacteria*, and *Chlorobi* isolated from a variety of environments (1, 21, 22).

Initially, it was suggested that integrons could be divided into two categories: the mobile integrons, which had few cassettes, usually encoding antibiotic resistance, had diverse *attC* sites and gained their mobility by association with transposons or plasmids, and the "superintegrons," which could have hundreds of cassettes, had homogenous *attC* sites and were located on chromosomes (9, 21, 23, 24). This distinction was based on a limited set of examples, and it is now clear that there is a continuum of integron structures between these two extremes (1, 25, 26). Nevertheless, the location of integrons on chromosomes versus mobile elements has important functional and evolutionary consequences, since mobility allows penetration into new taxa, while chromosomal locations can become sites for generating genomic complexity and phenotypic diversity (18, 27).

The clustering of integrons by environment (terrestrial versus marine), rather than by the identity of their host cells suggests that lateral transfer of integrons can occur between bacterial species residing in similar environments. This suggestion is supported by comparisons of phylogenetic trees based on the 16S rRNA gene or *rpoB* with those based on *intI*. The phylogenies are not congruent, and they demonstrate that lateral transfers of chromosomal integrons have occurred between bacterial groups (1, 21). In some cases, such as the chromosomal integrons found in *Shewanella*, *Xanthomonas*, and the *Vibrio cholerae* clades, the integron was clearly acquired before radiation of the extant species. However, in many other instances, closely related integrons are found in distantly related bacterial lineages. Two conclusions may be drawn: first, that the integron system is an ancient one, dating back hundreds of millions of years at least, and second, that over this evolutionary time there has been considerable lateral transfer of integron platforms between different lineages, even though in the short term some integrons are transmitted mainly vertically (1, 21).

Integrons are not mobile in their own right, since the integron integrase cannot excise its own gene from a chromosome. Rather, integrons must rely on linkage to transposases or recombinases for interchromosomal mobility. Chromosomal integrons often have such genes within their cassette arrays, or they lie adjacent to the integrase gene. Transposases are commonly found within *Xanthomonas* cassette arrays (18) and have been found adjacent to chromosomal integrons in *Comamonas* and *Pseudomonas* (28, 29). Recombinase genes lie adjacent to chromosomal integrons in *Azoarcus*, *Acidovorax*, and *Delftia* (30, 31), and overall some 30 out of 50 genome-sequenced integrons are linked to transposase or recombinase genes (1). In *Acinetobacter* and *Enterobacter*, some are flanked by miniature inverted-repeat transposable elements (MITEs). Recombination between these flanking regions or MITE-directed transposition can excise the integron-containing element from the chromosome (32, 33). Mapping of chromosomal class 1 integrons in betaproteobacteria has identified conserved, precise sequence boundaries for integron excision (see Fig. 4), although the enzymes responsible for this process are not known (34).

Diversity of Chromosomal Integrons

The first chromosomal integron to be described was from *Vibrio cholerae* (23, 35). It differed significantly from all integrons characterized up to that time because it was located on a chromosome and had hundreds of gene cassettes that encoded novel proteins of largely unknown function. Work over the last 15 years has now shown that chromosomal integrons are the norm for environmental bacteria, while the "clinical" integrons, borne on plasmids and typically found in pathogens, are a recent phenomenon driven by human antibiotic selection.

As more genome sequences of bacteria have been determined, it has become apparent that chromosomal integrons are a common feature of bacterial DNA. A recent survey showed that up to 17% of bacterial genomes in the NCBI database contained an integron integrase gene (2). Chromosomal integrons are most commonly found in various classes of *Proteobacteria* (beta through epsilon) but have also been reported in *Chlorobi*, *Cyanobacteria*, *Spirochaetes*, and *Planctomycetes* (1). As more genomes are sequenced, the range of species and phyla that contain integrons is likely to expand.

Integrons have been extensively studied in *Vibrio*, where all species investigated to date carry these elements on their chromosomes (36–39). The cassette arrays in *Vibrio* integrons are large, with reported arrays containing between 36 and 219 cassettes and accounting for between 0.7% and 3.1% of the genome (36). In general, integrons in vibrios are inherited vertically, with only two independent acquisitions of an integron platform identified by phylogenetic analysis, one into the *V. fischeri* group and one into *V. cholerae* and relatives (36, 39). The acquisition of an integron predated speciation in both these groups, and consequently integron activity and the assembly of large, diverse collections of gene cassettes have accompanied the evolution of the vibrios for hundreds of millions of years (21).

A chromosomal integron also seems to be an ancestral feature of *Xanthomonas*, since an integron integrase gene is located at the same chromosomal location, downstream from the dihydroxy-acid dehydratase gene, *ilvD*, in all strains tested (18, 40). The xanthomonad arrays are shorter than those in vibrios, having between 1 and 22 cassettes. Particular cassettes and arrays are associated with specific strains and pathovars, suggesting a role for those gene cassettes in conferring pathogenicity on particular host plants (18). The correspondence between individual cassette arrays and xanthomonad species, strains, and pathovars means that PCR typing of cassette arrays can be used for identification and epidemiological studies (41–44).

Chromosomal integrons are also present in *Pseudomonas*, although in this genus integrons are distributed patchily among the various species, suggesting multiple instances of acquisition by lateral gene transfer (1, 24). Cassette numbers are similar to those in xanthomonads, ranging from 10 to more than 32. The cassette arrays vary considerably in content, with otherwise identical strains sharing few or no gene cassettes. The ability of the integron

platform to capture and express gene cassettes has been formally established for at least some members of the genus (45, 46).

In the integrons described above, the integron integrase gene and the gene cassettes are transcribed in opposite directions (Fig. 1A and B). Some bacterial phyla with chromosomal integrons have the integrase gene transcribed in the same orientation as the cassettes (47). The best-studied phylum with this characteristic is the *Spirochaetes*, in particular the genus *Treponema*. Integrons in the ancestral *Treponema* appear to have been acquired in a single lateral transfer event and subsequently lost in some members of the genus. In the best-studied species, *T. denticola*, the number of cassettes in the respective arrays varies between 18 and 45 (22, 47). The cassettes in these arrays are extraordinarily dynamic. Examination of metagenomic data from the human microbiome project showed that the oral *Treponema* strains detected carried a total of 826 gene cassettes and that few of these elements were shared between individual strains (48).

Not all chromosomal integrons carry large numbers of gene cassettes. Examples of chromosomal integron integrase genes that are associated with just a few gene cassettes, or none at all, can be found in the genera *Shewanella*, *Nitrosomonas*, *Psychromonas*, *Oceanobacter*, *Geobacter*, *Pelobacter*, *Marinobacter*, and *Synechococcus*, to name a few (1). Whether these integrons are active in natural environments is not known, but the *Shewanella* and *Nitrosomonas* integrases are both functional (49, 50).

Chromosomal integrons as a source of genomic diversity. Chromosomal integron arrays are a hot spot for genome diversity (51). Even within closely related strains of a single bacterial species, different isolates can have very different gene cassette arrays. In *Treponema* and *Pseudomonas*, different isolates of otherwise identical strains often harbor few cassettes in common (22, 46, 48).

Most comparative analysis has been done in the vibrios, where acquisition, loss, and rearrangement of gene cassettes generate considerable diversity within serotypes, strains, and species (39, 52–55). There appears to be frequent movement of cassettes between vibrios, with high rates of loss or gain of individual elements. These rearrangements generate significant differences in cassette content and order (36). Movements of gene cassettes often seem to involve entire blocks within the array, thus mobilizing a series of linked cassettes in one event. The diversity thus generated can be used as a phylogenetic typing system for tracking pandemic strains (56, 57).

The speed with which diversity is generated in the *V. cholerae* integron cassette array allows repeated rearrangement and cross-species sampling of genes. This activity generates diverse genotypes that can then be acted upon by natural selection, allowing rapid adaptation to local conditions (27). In-depth analyses of 12 *Vibrio* spp. isolated from coral mucus showed that only 1 to 10% of the cassettes in their arrays were held in common. Even when isolates carried the same cassette, the position of that cassette in the array was different. Consequently, the arrays in some vibrio species may evolve even more quickly than those in *V. cholerae* (58).

So what triggers activity of the integron integrase gene and consequently leads to gene cassette rearrangements? Within the *intI* promoter region are binding sites for LexA, a transcriptional repressor that governs the SOS response. Induction of the SOS response triggers the expression of integron integrase and thereby increases cassette excision rates by orders of magnitude (59). This regulatory machinery appears to be ancestral, as it is preserved in both chromosomal and mobile integrons (60). Transformation with foreign DNA and bacterial conjugation both induce the SOS response and therefore upregulate integrase activity, as do stress and exposure to antibiotics (61–64). Integrase-mediated recombination also increases during the stationary phase (65). Consequently, the cassette acquisition and rearrangement machinery is stimulated at precisely the time when acquiring new functions and genetic diversity might be most advantageous.

However, unregulated integrase activity in stable environments runs the risk of rearranging cassette arrays that already have an optimum content and order. For this reason, we might expect that integrase activity is downregulated under such circumstances. Certainly, in *Xanthomonas*, where individual pathovars specialize as pathogens of particular plant species, there is widespread inactivation of the integrase gene by frameshifts, nonsense mutations, and deletions (18). In at least some cases, IntI activity brings about a fitness cost, particularly under stable conditions, and modeling suggests that episodes of selection during environmental perturbations are needed to help maintain functional integrase genes in bacterial genomes (66, 67). This may explain why almost one-third of IntI genes are inactivated and why there are frequent losses and gains of this gene within lineages (68). Taken together, these features show that integrons are uniquely placed to rapidly generate diversity in gene content and order during periods of change and natural selection, while remaining quiescent or becoming inactive during periods of environmental stability.

Gene Cassette Structure and Recombination

Gene cassettes are compact DNA elements that generally have a simple structure, consisting of a single open reading frame and a recombination site (7) (Fig. 3). The recombination site is called *attC* (shown in its integrated format in Fig. 3A) and exhibits significant internal homology that allows formation of stable secondary structures that are important both for recognition by IntI and for recombination (10) (Fig. 3B). Using Fig. 3A as a guide, from left to right, the typical features of a gene cassette in an array are as follows: the section of *attC* cleaved during insertion into the array and consisting of the conserved nucleotides TTRRRY; a short noncoding region, often less than 10 nucleotides, which may contain a ribosome binding site; a start codon (ATG, GTG, or TTG) for the internal open reading frame (ORF); a stop codon, often located in *attC*; and *attC* itself, consisting of a series of inverted repeats (R″, L″, L′, and R′) which are the integrase binding domains (10, 20, 69). Variations to this basic structure involve mainly the identity and orientation of the embedded open reading frame. Most cassettes contain a single ORF, oriented from left to right, but cassettes with two or more ORFs, no ORFs, or ORFs in reverse orientation are known (Fig. 1) (20, 70).

The *attC* sites are the recombination substrate recognized by the integron integrase, IntI. There are four integrase binding domains within *attC*, designated here, after the scheme of Johansson et al. (69), R″, L″, L′, and R′ (Fig. 3B). Among these binding domains, only R″ and R′ have conserved sequences, these being 5′-RYYYAAC and 5′-GTTRRRY, respectively. Despite the lack of sequence conservation, there is strong conservation of palindromic sequence elements within *attC*, such that it can fold into a cruciform secondary structure by pairing R″ with R′ and L″ with L′ (10, 71). Since the central part of *attC* exhibits considerable variation in sequence and length, it appears that conservation of the

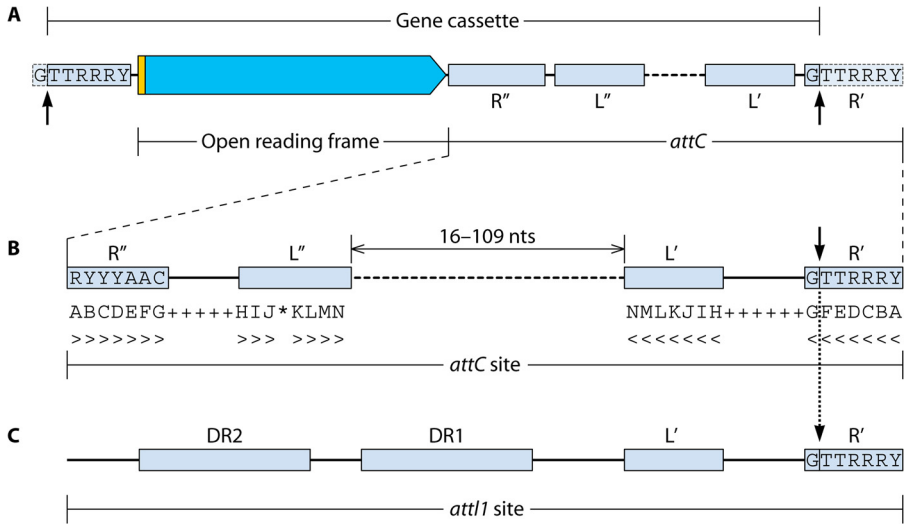

FIG 3 Structure of gene cassettes and associated recombination sites. (A) A single gene cassette is shown in linear form, inserted into a cassette array. From left to right, the salient features are as follows: the conserved recombination site, GTTRRY, with the vertical arrow showing the recombination point; the start codon and open reading frame encoded by the cassette; and the *attC* site, containing integrase binding domains R″, L″, L′, and R′. (B) Detailed structure of a single *attC* site. These elements have partially palindromic sequences (labeled with letters), such that R″ can pair with R′ and L″ can pair with L′, thus forming a stable cruciform structure recognized by integron integrases. An extra base, labeled with an asterisk in L″, ensures correct orientation and insertion of cassettes into the array. Between the terminal palindromic regions is a region that varies in length (16 to 109 nucleotides [nts]) and sequence between different cassettes. This region is also capable of forming a stable secondary structure, and the lack of sequence conservation suggests that structure, rather than sequence, is important for recognition. (C) An *attI* site from a class 1 integron. The *attI1* site also has L and R elements, with the conserved recombination point G↓TTRRRY. The *attI* of class 1 integrons also has two direct repeats, DR1 and DR2, but these are not known from the *attI* sites of other integron classes.

attC secondary structure is more critical for activity than the *attC* sequence (72). Indeed, it has been shown that IntI binds to the bulged hairpin DNA of the secondary structure (69). The protruding base in L″ (asterisk in Fig. 3B) serves to orient the polarity of the recombination event by determining which strand is recombined and thus ensures that cassettes are inserted in the correct orientation (2, 72). Recombination between *attI* and *attC* involves only the bottom strand of the incoming *attC*, and the single-stranded recombination structure is then resolved by replication (73, 74). Because IntI activity is dependent on structure, rather than sequence, this explains why diverse IntI proteins are able to mobilize gene cassettes with very different *attC* sequences (75).

The most common form of cassette insertion event involves recombination between *attC* and the integron-associated *attI* site (4). The *attI* site, like *attC*, carries integrase binding sites, called L and R (Fig. 3C). The R binding site contains the canonical sequence 5′-GTTRRRY, with incoming gene cassettes being inserted between the G and T residues. In the *attI* sites of class 1 integrons, there are two further integrase binding sites, consisting of direct repeats termed DR1 and DR2 (76, 77) (Fig. 3C). However, neither this region nor the L binding site sequence is conserved among *attI* sites of other integron classes. Different IntI proteins preferentially recognize their attendant *attI*, but they are able to operate on *attI* sites from heterologous systems, albeit with lower efficiency (78).

Integron integrases can also catalyze other recombination reactions in addition to *attI* × *attC*, although less efficiently (4, 79). Recombination between two *attC* sites in a cassette array excises cassettes as DNA circles (11). These circles may contain multiple gene cassettes, thus deleting or rearranging a block of linked cassettes (57). Insertion of cassettes into an array during *attC* × *attC* recombination is also possible, but *attI* is the preferred insertion point for incoming cassettes (80, 81).

Recombination between two *attI* sites is the least efficient integrase-catalyzed reaction (79) but may show an increase in frequency during late log phase and early stationary phase (65). Such recombination events could generate hybrid *attI* sites (82) and fuse different integrons into new arrangements. *attI* can also inadvertently recombine into secondary sites, which usually contain the conserved GTT motif characteristic of the *attI* recombination point. Insertion into secondary target sites via *attI* recombination fuses the integron into a new genomic location (82), and this may explain the movement of integrons between chromosomal locations.

attC sites can also recombine with secondary sites in the genome, and in such cases the target site may be the simplified recognition sequence GNT. These insertions render the *attC* inactive and thus fix the gene cassette at the secondary location. This may be an important mechanism for gene acquisition onto plasmids and chromosomes (83, 84). In other cases, when the secondary site has the canonical GTTRRRY sequence, nonspecific insertion retains the integrity of the *attC*, and thus the gene cassette can still be excised via integrase activity (85).

Expression of Gene Cassettes

Gene cassettes often only contain an open reading frame and an *attC* recombination site. This means they rely on an external promoter for expression. Most work on cassette expression has been conducted using the class 1 integron system, where expression of cassettes is driven by one of two promoters, Pc1, located in the IntI1 gene, and Pc2, located in *attI1*. A number of promoter variants that vary in strength have been identified (5, 6). Integrons with weaker promoters often have higher excision activity by the integron integrase (86).

Promoters within the integron integrase gene drive expression of gene cassettes in the associated array, but the strength of expres-

sion drops off as cassettes become more distal to the promoter (5, 87). This may be why the cassette arrays in clinical class 1 integrons rarely contain more than six cassettes, as any additional cassettes may not be expressed because of their distance from the promoter. In addition, the ability of *attC* sites to form stable stem-and-loop structures may impede ribosome progression along polycistronic RNAs, further reducing translation of polypeptides encoded by downstream cassettes (88).

Promoters that drive cassette expression have also been identified in the *attI* regions of class 2 integrons (6, 89) and within the *intI* genes of both class 3 integrons and the chromosomal integron of *Pseudomonas stutzeri* (5, 45). Consequently, it seems probable that all integrons carry a cassette promoter within the *intI-attI* region. However, some chromosomal cassette arrays carry hundreds of gene cassettes and are simply too long for expression of all the cassettes to be driven by a single promoter. Either these cassettes are transcriptionally silent or they carry their own internal promoters. In the very long cassette arrays that characterize many *Vibrio* species, it appears that most cassettes are transcribed and that this transcription is enhanced by some stress treatments (90). Consequently, it seems likely that gene cassettes with internal promoters are scattered throughout the *Vibrio* arrays and that these help drive the expression of downstream cassettes.

Internal promoters have been identified in a number of gene cassettes. The *cmlA* chloramphenicol resistance gene cassette has its own promoter (91, 92), and it appears that the quinolone resistance genes of the *qnrVC* family may all carry internal promoters (93). This arrangement would allow expression of these cassettes regardless of their position in the array. Toxin-antitoxin (TA) genes are a common feature of large chromosomal cassette arrays and are thought to contribute to array stability (94, 95). To maintain themselves in lineages, such genes must be constitutively expressed, so it is not surprising that such cassettes also have their own promoters (39, 96). Finally, a number of cassettes have been identified in metagenomic samples that have no identifiable open reading frame. These ORF-less cassettes may be mobile, cassette-borne promoters (20, 70).

Gene Cassette Diversity and Function

Integron gene cassettes are common, and abundant, in environmental samples. Metagenomic analyses show that gene cassettes can be recovered from every environment that has been investigated: desert soil, forest soil, polar soil, riverine sediment, hot springs, and estuaries (20, 70); seawater, marine sediment, and deep sea vents (15, 16, 20, 97, 98); and biofilms, plant surfaces, and the symbionts of eukaryotes (15, 17, 18, 34). Thus, cassettes are widely disseminated in diverse environments. Cassettes also have diverse origins, since homology and codon usage analyses show that the ORFs carried by gene cassettes originated in diverse bacterial phyla (1, 21, 97).

Gene cassettes are an enormous reservoir of genomic novelty (70). Combined analyses of metagenomic and chromosomal gene cassettes show that up to 65% of cassettes and their encoded polypeptides have no known homologues in DNA or protein databases. A further ~15% exhibit homology to conserved hypothetical proteins, while the remaining ~20% have sufficient homology to characterized proteins that their function might be predicted (1). Many cassette-encoded polypeptides are predicted to form novel protein folds and thus may comprise a toolbox of flexible molecular components for assembling new quaternary structures (99, 100). The composition of gene cassettes in environmental samples exhibits significant spatial turnover, even across distances as small as one meter (19). Given this, it is not surprising that different environments have distinctive populations of gene cassettes and that there is often little overlap in composition between environments (15, 16, 97, 98).

The mobile nature of gene cassettes means that their genomic locations and host cells are not fixed, and this creates problems for conventional annotation. Consequently, a number of dedicated databases have been established for annotation and curation of integrons and their gene cassettes (101, 102). Software for identifying cassettes in DNA sequences has been developed (39). Because some research groups focus more on the clinical aspects of integron biology, databases and annotation systems that deal exclusively with gene cassettes from integrons found in pathogens have also been developed. These cassettes mainly encode antibiotic resistance (103–105).

Some gene cassettes recovered from metagenomic DNA or chromosomal integrons do not appear to encode polypeptides (20). Such noncoding cassettes can make up a considerable proportion of arrays. For instance, they comprise between 4 and 49% of *Vibrio* cassette arrays (36). These noncoding cassettes might encode promoters or regulatory RNAs. In *Xanthomonas campestris* pv. *campestris*, the *trans*-acting small RNA (sRNA) Xcc1 is encoded by an integron gene cassette and is involved in regulation of virulence (106). In metagenomic DNA, a family of noncoding gene cassettes that demonstrates conservation of a central motif with an imperfect inverted repeat has been recovered, suggesting that RNA structure rather than sequence is important (70), which again is a feature of regulatory RNAs.

The functions of about 20% of environmental gene cassettes can be inferred through homology with known genes. These functions are diverse and include secondary metabolism, plasmid maintenance, virulence, and surface properties. Toxin-antitoxin (TA) systems are commonly found within or adjacent to integrons (1, 2, 20, 21, 39). Loss of TA systems kills the cells that housed them, since the toxin has a longer half-life than the antitoxin that inactivates it. Consequently, the presence of TA systems within integrons may stabilize chromosomal arrays and maintain integron-bearing plasmids within cells (94–96, 107).

A number of gene cassettes appear to encode functions associated with virulence and host relationships, including lipocalin (108), capsular polysaccharide (109), enterotoxin (110), isochorismatase (97), lipases (70, 111), and methionine sulfoxide reductases (32). The diversity of cassettes with other inferred functions is impressive, covering DNA modification, functions related to phage, polysaccharide biosynthesis, amino acid synthesis, transporters, and efflux systems, to name a few (1, 17, 21, 112, 113). The presence of signal peptides for export to cell membranes implies that cassette gene products are often important for interacting with the local environment and help to create surface properties necessary for biofilm formation or for interactions with phage and grazers (39, 97, 114).

The conclusion must be reached that the gene cassettes contained within integrons are an important component of bacterial adaptation. The ability of integrons to acquire new gene cassettes, and to rearrange those already within arrays, provides a rapid means of generating adaptive diversity (58). These conclusions are confirmed by observations of bacterial adaptation in natural environments. The genus *Xanthomonas* is specialized for pathoge-

nicity on plants. Different pathovars are restricted to different plant hosts, and in each case their integron cassette arrays are unique (18). Integrons help bacteria adapt to particular niches (16, 115), encode functions relevant to interactions with symbionts (15), and may help generate ecotypes (27). In polluted marine sediments, cassettes encode diverse functions relevant to catabolism of industrial waste, such as polypeptides dealing with the transport and catabolism of aromatic compounds (98).

Clearly, integrons and their gene cassettes are an important resource for bacterial adaptation. Given this, it is not surprising that integrons have had a major role in the adaptation of bacteria to antibiotic therapy. Because integrons have access to a vast pool of gene cassettes with diverse functions, they were preadapted for acquisition and expression of resistance determinants, allowing integron-containing cells to rapidly fix under the strong selection pressure imposed by antibiotic use. Environmental gene cassettes that can be, and have been, coopted as resistance determinants have been described, such as the various efflux pumps encoded by the *qac* gene family (17). In some cases, potential antibiotic resistance activity has been demonstrated for novel gene cassette products. These include an RNA methyltransferase and a phosphotransferase (116). A number of cassettes recovered from chromosomal integrons have significant homology to known antibiotic resistance genes (9), and various gene cassettes from *Vibrio* can be demonstrated to confer resistance phenotypes (117–120).

Thus, prior to the antibiotic era, integrons were already poised to take advantage of their access to the vast pool of genetic novelty encoded by environmental gene cassettes. The use of antibiotics in human medicine and agriculture then provided the selective force to fix rare events where integrons had acquired gene cassettes of relevance to antibacterial resistance. This sampling of the resistome (121, 122) has continued to the present day, resulting in the accumulation of gene cassettes with diverse mechanisms for dealing with an equally diverse number of antibiotics. The history of this evolution is the subject of the next section of this review.

INTEGRONS IN THE PRESENT: THE RISE OF ANTIBIOTIC RESISTANCE

Integrons are major players in the spread of antibiotic resistance, particularly in Gram-negative pathogens. In resistance integrons, the functional integron platform is linked to mobile DNA elements such as transposons and/or conjugative plasmids, thus enhancing transfer between cells and species (21). There are five classes of "mobile" integrons, all associated with antibiotic resistance: classes 1, 2, and 3, usually recovered from clinical contexts (123); class 4, found on the SXT element of *Vibrio cholerae* (124); and class 5, found on the pRSV1 plasmid of *Alivibrio salmonicida* (125).

These integrons share a pool of gene cassettes, the majority of which encode resistance to antibiotics. In total, about 130 different resistance gene cassettes have been identified, whose diverse patterns of codon usage and heterogeneous *attC* sites strongly suggest that they have been accumulated incrementally from diverse phylogenetic backgrounds (2, 21, 103). Cassette arrays in mobile integrons are usually short, with the longest recorded array having eight cassettes (126), presumably because cassette expression is driven from a single promoter, and proximal cassettes are poorly expressed (5). The pool of cassettes carried by mobile integrons can confer resistance to most classes of antibiotics used in medicine and agriculture (103).

Antibiotic resistance integrons have a number of features in common. They are usually mobile, and their cassettes arrays are short and normally encode antibiotic resistance. However, these shared features are not intrinsic properties of their ancestor integrons but have arisen as a result of convergent evolution, driven by the strong selection pressures imposed during human antibiotic use. Each of the major classes of integron now found in antibiotic-resistant pathogens has a similar, and recent, evolutionary history.

Origin of Class 1 Integrons as Vectors for Antibiotic Resistance

Chromosomal class 1 integrons have been found in a wide range of nonpathogenic *Betaproteobacteria*, including members of the genera *Hydrogenophaga*, *Aquabacterium*, *Acidovorax*, *Imtechium*, *Azoarcus*, and *Thauera*. These chromosomal integrons carry gene cassettes of unknown function and exhibit significant sequence diversity in their IntI1 genes (30, 34, 127). All chromosomal class 1 integrons characterized from environmental sources have common terminal sequences. The conserved left-hand breakpoint occurs exactly 107 bp beyond the IntI1 stop codon (34), at the same position where the ISPa7 element inserts into class 1 integrons carried by some *P. aeruginosa* isolates (128). The right-hand breakpoint is also conserved, occurring 43 bp beyond the final *attC* element (Fig. 4). The presence of such precise breakpoints in a diversity of chromosomal landscapes and in a range of different species is strong evidence that the chromosomal class 1 integron is comparatively mobile, even over short evolutionary time frames, and that site-specific recombination is involved (34). The mechanism for mobilizing the integron is unknown.

Examination of bacteria from soil, freshwater, and biofilms suggests that 1 to 5% of cells may carry a class 1 integron (129, 130). In some environments, this proportion may rise to as high as 30% (34). Class 1 integrons in the environment exchange gene cassettes in a dynamic fashion (17), and the class 1 integron integrase is able to access gene cassettes from other classes of chromosomal integrons (89, 120). Class 1 integrons from biofilms and freshwater often have cassettes carrying *qac* genes, a gene family that encodes versatile efflux pumps (17, 129, 131).

In summary, the chromosomal class 1 integrons found in environmental bacteria were preadapted and exquisitely positioned to respond when humans attempted to control bacterial growth with antibacterial agents: they were abundant in bacteria that occupy diverse environments intersecting with the human food chain; they were comparatively mobile, moving between chromosomal locations and species; they were able to sample gene cassettes from the extraordinarily diverse cassette arrays held by environmental organisms; and they commonly carried gene cassettes for efflux pumps capable of conferring resistance phenotypes.

While the actual sequence of events may never be precisely known, we have enough information to reconstruct the likely evolutionary history of the mobile class 1 integrons that are now a major factor in the dissemination of antibiotic resistance (Fig. 5). Examination of metagenomic DNA recovers diverse class 1 integron integrase genes, many with less than 95% nucleotide sequence identity (127). In contrast, the integron integrase genes in all class 1 integrons from clinical sources are essentially identical, strongly suggesting that all clinical class 1 integrons are recent descendants of a single event involving just one representative of

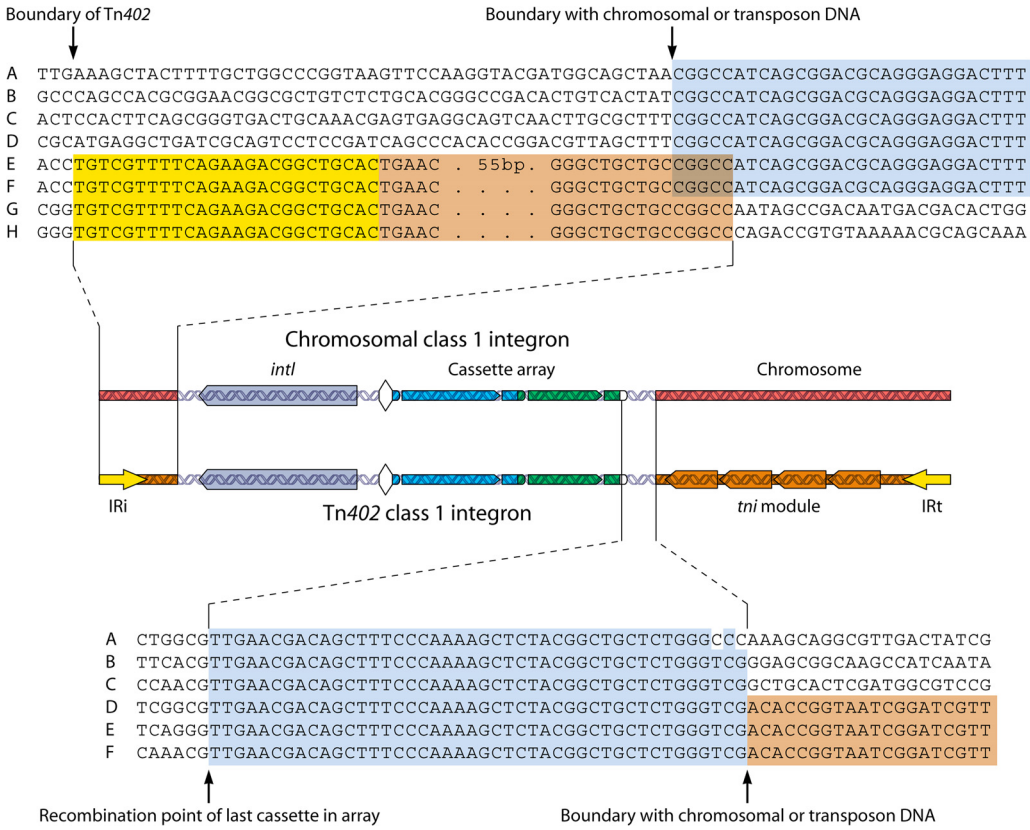

FIG 4 Conserved sequence boundaries of chromosomal class 1 integrons. Schematic maps of a chromosomal class 1 integron as found in betaproteobacteria and after its insertion into a Tn*402* transposon are shown. Symbols are as Fig. 1 and 2. Additional features: IRi and IRt are the 25-bp terminal inverted repeats of the Tn*402* transposon, and the *tni* module contains genes involved in Tn*402* transposition activity. Both the left- and right-hand boundaries of the class 1 integron demonstrate precise sequence breakpoints. Sequences in the top alignment, showing the left-hand boundary, include relevant regions from the chromosomal class 1 integron from *Hydrogenophaga* PL2G6 (accession no. EU327989) (A), the chromosomal class 1 integron from *Aquabacterium* PL1F5 (accession no. EU327988) (B), the chromosomal class 1 integron from *Acidovorax* MUL2G8 (accession no. DQ372710) (C), the chromosomal class 1 integron from *Imtechium* PL2H3 (accession no. EU327990) (D), the IncP-1 beta multiresistance plasmid pB8, which also carries Tn*402* (accession no. AJ863570) (E), plasmid R751 from *Enterobacter aerogenes*, which carries Tn*402*, and a clinical-type class 1 integron contained within this transposon (accession no. U67194) (F), Tn*6008* from *Enterobacter cloacae*, which carries sequence typical of Tn*402*-like transposons up to the CGGCC motif shared with class 1 integrons but carries no class 1 integron sequences beyond that point (accession no. EU316185) (G), and a bla_{VIM-1} clinical class 1 integron from *Pseudomonas aeruginosa* VR-143/97 that has an ISPa7 insertion element inserted at the Tn*402*/class 1 integron boundary (accession no. Y18050) (H).

the diverse *intI1* sequence variants present in natural environments. The fact that *intI1* sequences identical to those now found in clinical pathogens can also be found on the chromosomes of nonpathogenic environmental *Betaproteobacteria* adds significant weight to this conclusion (34).

The evolutionary history of the clinical class 1 integron is thought to be as follows. An integron located in a betaproteobacterium from a biofilm or freshwater environment excised from its host chromosome at the precise boundaries outlined in Fig. 4. This integron was then captured by a Tn*402*-like transposon, probably via site-specific recombination. Examples of candidate Tn*402*-like elements, prior to the acquisition of a class 1 integron, have been found in human commensal flora (34). The newly formed hybrid element then consisted of a class 1 integron integrase and attendant cassettes embedded within a typical transposon, still carrying inverted terminal repeats and full transposition machinery (Fig. 5). Most clinical class 1 integrons have now lost the Tn*402* transposition functions, but it seems almost certain that the original capture event involved a functional transposon. In support of this idea, Tn*402*-type class 1 integrons that retain the full Tn*402* transposition machinery but lack any antibiotic resistance gene cassettes can be recovered from environmental sources (28, 29, 132). It is therefore probable that prior to human antimicrobial use there were any number of integron/transposon hybrids circulating in the environment, of which the Tn*402*-integron is the most successful descendant.

Part of the success of the Tn*402*-integron may lie in an unusual and adaptive property of Tn*402*. It specifically targets and transposes into the *res* sites of plasmids (133). Consequently, a class 1 integron embedded in a Tn*402* transposon would soon find itself on a diversity of different plasmid vectors, thus enhancing its ability to disseminate between bacterial cells and species. Indeed, one of the most successful of these insertion events involved transposition of the Tn*402*-integron into a mobile element that contained the *mer* operon, encoding resistance to mercury. The resulting compound element, Tn*21*, has itself come to be widely distributed in different plasmids and to spawn a series of complex and compound derivatives (134, 135).

The selective forces that first fixed the Tn*402*-class 1 integron in a human commensal or pathogen cannot be known with cer-

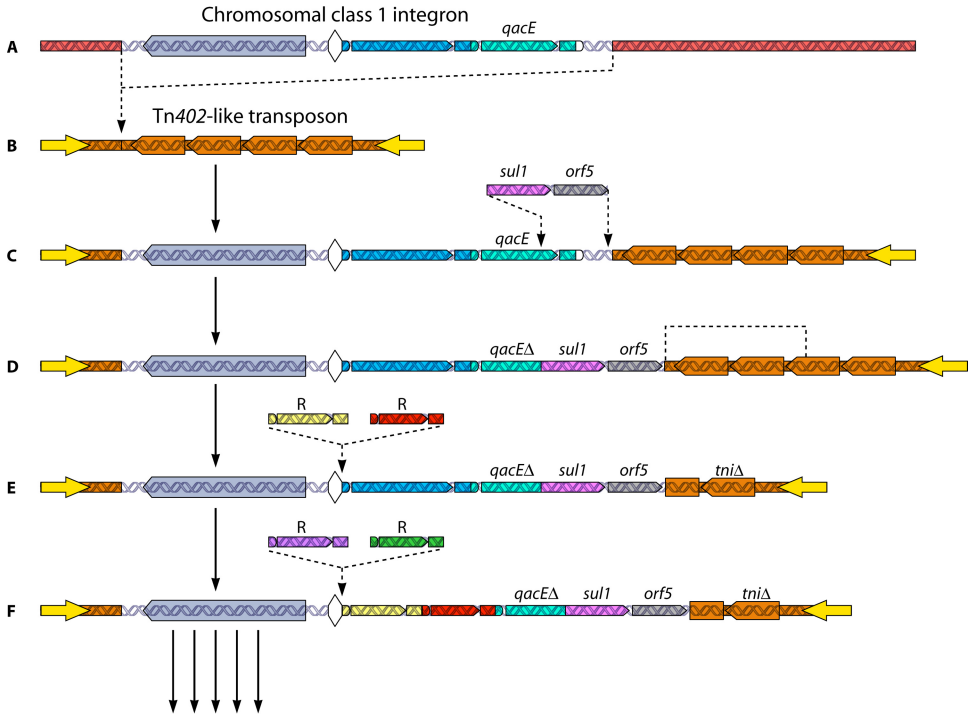

FIG 5 Model for the origin and subsequent divergence of the mobile class 1 integrons that are now common in Gram-negative pathogens. (A) The common ancestor of all clinical class 1 integrons was a member of a diverse group of class 1 integrons located on the chromosomes of *Betaproteobacteria*. (B and C) This chromosomal class 1 integron was captured by the Tn*402* transposon (B) to generate a transposon/integron hybrid carrying the *qacE* cassette, encoding resistance to disinfectants (C). (D) A gene for resistance to sulfonamides, *sul1*, was then captured, deleting part of the *qacE* cassette and thus generating the 3′ conserved segment (3′-CS). (E) Deletions and insertions involving *tni* generated Tn*402* transposition-incompetent integrons, while acquisition of further antibiotic resistance cassettes took place, expanding the range of antibiotic resistance phenotypes conferred by integrons. (F) Acquisition of new cassettes continued, and the Tn*402*-integron hybrid moved onto diverse plasmids and other transposons, such as the Tn*21* family. These events generated further diversity and accelerated the penetration of class 1 integrons into a wide variety of pathogens and commensals.

tainty. However, circumstantial evidence strongly points to a role for *qac* gene cassettes. These genes encode versatile efflux pumps that confer resistance to toxic cationic molecules such as quaternary ammonium compounds (131) and may have a role in defending cells against toxins found in natural ecosystems (136). They are found in about half the cassette arrays carried by class 1 integrons recovered from natural environments (113), and *qac* cassettes are dynamically exchanged between integrons in freshwater biofilms (17). Consequently, there is a 50% chance that any class 1 integron inserted into a Tn*402* backbone would carry a *qac* cassette. Such an integron would confer resistance to quaternary ammonium compounds, providing a significant advantage to cells carrying the Tn*402*-integron and driving them to fixation in human-associated bacteria exposed to these disinfectants (34, 113). Quaternary ammonium compounds were first used as hospital disinfectants in the early 1930s, predating the clinical use of antibiotics (137). This would explain why the possession of the *qacE* gene appears to be ancestral in clinical class 1 integrons (138).

The first true antibiotics were the sulfonamides, introduced during the mid- to late 1930s (139). Selection for antibiotic resistance begins from this point, so it is not surprising that the next event in the evolution of clinical class 1 integrons involves a gene for sulfonamide resistance. The *sul1* gene encodes a drug-resistant variant of the sulfonamide target enzyme, dihydropteroate synthase. This gene was inserted into the Tn*402*-class 1 integron, deleting the end of the *qacE* gene and its attendant *attC* (140), generating the 3′ conserved segment (3′-CS) that is characteristic of many extant clinical class 1 integrons (Fig. 5) (141). Various further deletions to the Tn*402* element led to the loss of transposition functions (142) and generated diversity in the 3′ end of the Tn*402*-class 1 integrons (135, 143).

The Tn*402*-class 1 integron was now firmly embedded in the human microbiota and free to sample gene cassettes held on chromosomal integrons. The class 1 integron integrase is readily able to recruit gene cassettes from other classes of integron (46, 89, 120), and where these cassettes confer antibiotic resistance, there is a strong selective pressure to fix the newly generated cassette arrays. Over time, the Tn*402*-class 1 integrons have acquired gene cassettes that confer resistance to the majority of antibiotic classes used to control Gram-negative bacteria (2, 21, 144). In all, some 130 different antibiotic resistance gene cassettes from clinical class 1 integrons are now known, along with a few other gene cassettes of unknown function (103).

The general ability of integrons to sample gene cassettes, coupled with the linkage between a class 1 integron and a plasmid-hunting transposon, has made the descendants of the original Tn*402*-class 1 integron insertion event into an extraordinarily successful family of mobile elements. They have readily spread by conjugation and natural transformation (145, 146), such that they are now found in some 40 to 70% of Gram-negative pathogens from clinical contexts (147, 148). They are common in the pathogens and commensal flora of livestock and companion animals

(144, 149, 150). They have also made their way into plant pathogens (151) and Gram-positive organisms (152). Their general abundance in human-dominated ecosystems and their release via human waste streams means that clinical class 1 integrons are increasingly being reported as "pollutants" of natural environments (144, 153).

The story of the class 1 integron is a salient lesson about the immense power of natural selection, especially when applied to organisms with large population sizes, rapid generation times, and access to a vast pool of genetic novelty. The clinical class 1 integron will continue to accumulate new gene cassettes encoding antibiotic resistance and other adaptive phenotypes and will continue to participate in new rearrangements with transposons, plasmids, and other mobile elements.

Origin of Class 2 Clinical Integrons

The class 1 integron is responsible for most reports of integron-mediated antibiotic resistance. It is associated with the greatest diversity of gene cassettes, is found in increasingly complex mosaic mobile elements, and is found in a very broad range of species. Nevertheless, other classes of integron that confer antibiotic resistance have also been described from clinical contexts. Class 2 and class 3 integrons share their cassette pool with the class 1 integrons but are distinguished by having divergent integron integrase sequences. Class 2 and class 3 integrons from clinical contexts also share a similar evolutionary history with the Tn*402*-class 1 integron, as both have probably been recruited onto transposable elements from a chromosomal ancestor. The history of these elements is outlined below.

Class 2 integrons are associated with the Tn*7* transposon (154, 155), whose transposition activity is directed at specific attachment sites on chromosomes or plasmids (156). Metagenomic studies have detected potentially functional class 2 integron integrase genes in agricultural habitats, associated with diverse *Firmicutes* and *Bacteroidetes* (157), and a functional class 2 integron from *Providencia stuartii* has been described. The latter integron carried an array of gene cassettes of unknown function, as might be expected for an environmental integron (158). In contrast, the integron integrase genes of class 2 integrons isolated from clinical contexts are inactivated by an internal stop codon (159), and their associated cassette arrays encode antibiotic resistance determinants. The fact that clinical class 2 integrons all carry the same internal mutation in the gene for IntI2 strongly suggests that they are all descendants of a single event.

Because the integron integrase gene of clinical class 2 integrons is inactive, this restricts the ability of the integron to acquire and rearrange gene cassettes. It is therefore not surprising that their cassette arrays are highly conserved (160) and that their range of cassette functions is much more limited than those of class 1 integrons (161–163). There are some variant cassette arrays described, and it is thought that these cassette rearrangements were mediated by integrase activity in *trans* or by suppression of the internal stop codon. It has been demonstrated that the class 1 integrase can recognize the class 2 recombination site, *attI2* (78, 159), so exposure of clinical class 2 integrons to the activity of other integron integrases might explain the observed diversity in class 2 cassette arrays.

Like the class 1 clinical integrons, class 2 integrons from clinical contexts have made their way into a diverse range of pathogens, commensals, and environmental microorganisms (162, 164) and have also been described from domesticated and wild animals (144). It appears that a second variant of the class 2 integron integrase might have been recently recruited onto an IncP plasmid in uropathogenic *Escherichia coli*. This *intI2* has six sequence differences from the *intI2* found in association with Tn*7*, including a glutamine codon (CAA) that removes the stop codon characteristic of other clinical class 2 integrons. The IntI2 encoded by this gene is capable of recombination reactions, and its cassette array carries a gene for trimethoprim resistance, *dfrA14*, that is more usually associated with class 1 integrons. In addition, it carries an unusual cassette, potentially encoding a lipoprotein signal peptidase, which may be of relevance for pathogenicity (165). Thus, integrons are still being recruited from the environmental pool of such elements and will continue to accumulate new gene cassettes relevant to resistance, pathogenicity and virulence.

Origin of Class 3 Clinical Integrons

Class 3 integrons were first described from clinical contexts in Japan (166) but, like the class 1 and 2 integrons, also had their origins in environmental bacteria. Typical chromosomal integrons with class 3 integrases have been characterized in two species of *Delftia* (31). These integrons have related cassette arrays that encode proteins of unknown function. They share features with chromosomal class 1 integrons from *Acidovorax*, including homology of a flanking recombinase gene and an identical endpoint at the boundary of insertion into chromosomal DNA (30, 31). Such chromosomal class 3 integrons can share gene cassettes with environmental organisms that carry class 1 integrons, since an identical gene cassette has been found in the *Delftia* integron and in a chromosomal class 1 integron cassette array from *Pseudomonas* (29).

Class 3 integrons from clinical contexts are associated with antibiotic resistance and have an evolutionary history similar to that of the class 1 integrons. The class 3 integron platform also appears to have been captured by a Tn*402* transposon, but in the reverse orientation to the class 1 capture event (167). Such class 3 integrons are relatively common in Japan, where they have spread into a number of human pathogens and commensals (168, 169). They are not commonly recovered elsewhere in the world (148, 170, 171) and do not carry a great diversity of gene cassettes, perhaps because the class 3 integron integrase is not as active as those of the other classes (78). The class 3 clinical integron is continuing to evolve, colonizing new species, acquiring novel resistance cassettes, and making its way onto new plasmid vectors (168, 172, 173).

Summary of the Current State of Resistance Integrons

Mobile integrons have been a major driver in the spread of antibiotic resistance, particularly among Gram-negative bacteria (174). Integrons have accumulated large numbers of resistance genes from the environmental pool of these determinants (122). They have also increased enormously in abundance, thus raising the possibility of interactions with other DNAs and of generating new and ever more complex mobile elements that carry resistance to multiple antibiotic classes, disinfectants, and heavy metals (175). Their evolution is ongoing, driven by the constant exposure to selective agents in both human-dominated and natural environments, with the result that they will continue to accumulate genes that confer advantageous phenotypes (153). The potential

outcomes of this evolution and its possible consequences for human welfare are discussed in the next section.

INTEGRONS IN THE FUTURE

Over the last 50 years, the widespread use of antibiotics has imposed strong selection for the assembly of mosaic DNA elements carrying multiple resistance genes. These DNA elements have made their way into diverse bacterial hosts, both commensals and pathogens, which have, in turn, colonized humans, their companion animals, and their domesticates. The result is that integrons, their antibiotic resistance genes, and the mobile DNA elements they reside upon have become widely distributed, highly diverse, and abundant in human-dominated ecosystems.

Understanding the natural activities and more recent evolution of integrons gives us some power to predict the likely future of these elements and to explore how their properties might be exploited. Integrons are exquisitely positioned to sample and express potentially any gene in the biosphere and to do so without perturbing existing genes. Consequently they have the power to promote adaptation to changing environmental conditions by rapidly generating genetic variation. This allows integron-containing cells to overcome human strategies for controlling bacterial growth, but it also offers rich opportunities for gene prospecting and construction of new biosynthetic pathways.

Integrons and Resistance Genes as Pollutants

The ongoing use of antibiotics in clinical and agricultural practice has made mobile resistance integrons extraordinarily abundant. The class 1 clinical integrons are particularly widespread, occurring in anywhere from 10 to 50% of commensal bacteria in healthy human subjects (176–178), including infants who have not yet been exposed to antibiotics (179). They are also present in the commensal bacteria of farm animals, where the integron carriage by commensal *E. coli* can rise to 80% (179–181). These commensal bacteria house integrons with diverse structures and act as a conduit for lateral gene transfer of resistance determinants between environmental bacteria, other commensals, and pathogens (182, 183).

Because the rate of integron carriage is so high in humans and their agricultural animals, large numbers of bacteria containing integrons and resistance genes are shed into the environment via waste streams. One estimate suggests that 10^{19} bacteria containing class 1 integrons are released in the United Kingdom each year, just via disposal of sewage sludge (184). As a consequence, integrons can be readily detected in wastewater treatment plants (164, 185). Resistance genes and integrons are present in floc and sewage sludge (186, 187), and despite the growing use of methods to remove such genes during wastewater treatment (188–191), considerable quantities are released in reclaimed water (192) or directly into rivers (193, 194), where they eventually make their way to estuaries and the ocean (171, 195, 196). Resistance genes and integrons are also disseminated in effluent from hospitals and in wastewater from tanneries (197, 198). Further, the use of animal wastes as manure introduces resistance genes and integrons into agricultural soils (199–202).

As a consequence, there is a zone of enrichment with clinical integrons and resistance genes that spreads out from human settlements (130, 132, 157, 203). This influence is so pervasive that integrons and resistance genes can now be found in situations far removed from antibiotic use, such as in remote communities (204, 205), the Arctic (206), and endangered species (207). The list of wild animals and natural environments where clinical integrons have been detected continues to grow (144), and levels of antibiotic resistance genes in soils have been increasing since the 1940s (208).

Antibiotic resistance genes and integrons are now viewed as significant environmental contaminants and as markers for tracing sources of pollution (209, 210). The resistance genes and integrons emanating from human-dominated ecosystems can be regarded as xenogenetic pollutants, because these DNA elements have been assembled under the continuous selection exerted by human antibiotic use. However, unlike conventional pollutants, integrons and resistance genes can replicate and therefore have properties of both pollutants and invasive species (153, 175). The human health implications of pollution with resistance genes has been the subject of considerable scrutiny (211, 212), but less attention has been paid to their potential effects on natural environments (153, 213).

Pollution with Selective Agents

When humans or animals are given antibiotic therapy, between 30 and 90% of the ingested compound is excreted to pollute wastewaters (214). Antibiotics are also released in large quantities from pharmaceutical plants (215, 216) and are spread during manuring (217, 218). Like resistance genes, antibiotics are difficult to remove during water treatment, and some have long half-lives in the environment (219, 220), resulting in pollution of both rivers and the ocean (221, 222). The use of antibiotics in aquaculture adds these compounds directly into water bodies (223, 224), raising local antibiotic concentrations. The environmental and health consequences of contaminating water bodies with antibiotics are of significant concern (225–227), with calls to monitor and control antibiotic pollution (121, 228).

Selection in Natural Environments

Human waste streams release integrons simultaneously with the antibiotics that select for carriage of integrons and resistance genes. Waste streams also carry significant quantities of other selective agents, such as disinfectants and heavy metals. Thus, resistant bacteria, their mobile elements, and their resistance genes are released into an environment containing significant quantities of selective agents and environmental organisms. As a consequence, wastewater treatment facilities and other water bodies become giant reactors for interaction between bacterial species, mobile elements, and their accessory genes (153). Transformation and conjugation induce the SOS response and promote integron recombination events, thus coupling the generation of diversity with lateral gene transfer (61, 62). Consequently, in such environments, lateral gene transfer is promoted and there are opportunities for complex interactions between various mobile elements. These interactions can generate ever more complex mosaic molecules that carry a growing armory of genes encoding resistance to diverse selective agents (229–232).

As the number of accessory genes linked to a particular mobile element becomes larger, so the potential for positive selection also increases. This is because exposure to any one selective agent provides an advantage for the whole DNA element, simultaneously selecting for all genes on the element through "hitchhiking" by simple linkage. As an example, selection for resistance to quaternary ammonium compounds is thought to have fixed the clinical

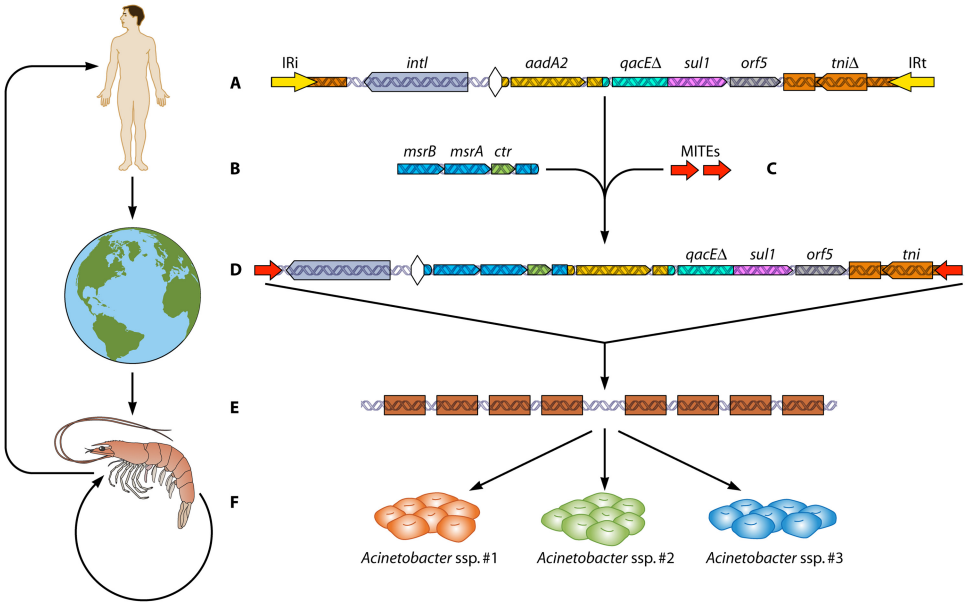

FIG 6 Role of resistance gene pollution in generating novel, complex DNA elements. (A) A typical class 1 integron from human-pathogenic or commensal bacteria. This type of DNA element commonly pollutes aquatic environments. It consists of inverted DNA repeats IRi and IRt, the class 1 integron integrase gene *intI1*, and a gene cassette, *aadA2*, which confers streptomycin resistance. The 3′ conserved segment consists of fused genes for disinfectant and sulfonamide resistance (*qacEΔ*/*sul1*), ORF5, and the remnants of genes encoding transposition functions (*tniΔ*). (B and C) In an aquatic environment, such an integron was modified by acquiring a novel gene cassette encoding two methionine sulfoxide reductases (*msrB* and *msrA*) (B) and replacing the inverted repeats IRi and IRt with miniature inverted-repeat transposable elements (MITEs) (C). (D) This event generated a compound MITE/integron element. (E) Mobility conferred by the MITEs allowed insertion of the compound integron into a genomic island. (F) This genomic island moved into at least three different species of the genus *Acinetobacter*, carrying the integron with it. Consequently, resistance determinants released from human waste streams may interact with gene cassettes and mobile DNA elements in aquatic ecosystems to generate new combinations of potential virulence genes in environmental bacteria. The presence of these bacteria in food items provides a readily accessible route for contamination of the food chain and the emergence of novel, virulent pathogens.

class 1 integron in a human commensal bacterium (113), and these disinfectants are known to coselect for elements carrying antibiotic resistance genes (129). Similarly, exposure to heavy metals can coselect for antibiotic resistance when resistance genes are carried on mobile DNA elements that also carry genes for resistance to those heavy metals (28, 233–235). In environments containing diverse resistance elements and diverse selective agents, plasmids can acquire genes for resistance to multiple antibiotics, disinfectants, and metals and at the same time assemble genes for degradative pathways capable of acting upon other xenobiotics (236). For these reasons, aquatic environments are regarded as a natural reactor for the generation of novel xenogenetic DNA elements (146, 153, 228, 237).

Generation of New DNA Elements and Newly Resistant Species

Pollution of natural environments with antibiotics and disinfectants affects community structure and leads to increased carriage of resistance genes in environmental organisms (238, 239). It is now widely accepted that the natural environment is a recruitment ground for resistant organisms and potential opportunistic pathogens (121, 144, 213, 240).

Continued copollution with clinical integrons and selective agents will lead to an increased abundance of resistant cells in the general environment and place additional selective pressures on environmental organisms. Two general trends might be predicted: that new opportunistic pathogens with resistance to antibiotics will arise and that their integrons will accumulate additional genes with effects on transmission, pathogenicity, and virulence. These trends can both be illustrated using recent observations of clinical integrons detected in marine organisms (Fig. 6).

If resistant bacteria are shed into natural environments, one might expect that they would be taken up by filter feeders. In light of this prediction, the integron status of edible prawns along the east coast of Australia was investigated. Over 75% of prawns collected from retail outlets tested positive for class 1 integrons. Detailed characterization of these elements showed that they had all the hallmarks of integrons originating from clinical contexts: 100% sequence identity to the clinical IntI1 gene, the presence of the 3′ conserved segment, and the presence of a typical gene cassette containing the antibiotic resistance gene *aadA2*. These data establish that the integron was ultimately derived from a human pathogen or commensal organism (32).

However, during its dissemination back onto the environment, the integron had undergone further evolutionary changes. It was now resident in *Acinetobacter johnsonii*, a species not normally associated with humans, and so must have undergone lateral gene transfer into its current location. In addition, the integron had acquired an unusual gene cassette that encoded two methionine sulfoxide reductases. These enzymes repair proteins damaged by oxidative stress and are likely to enhance colonization and survival within animal tissues. Finally, the termini of the original integron had been replaced by miniature inverted-repeat transposable elements (MITEs), potentially giving the integron a mechanism for mobility (32). Consequently the predicted trends outlined above have been confirmed: the class 1 integron had acquired additional gene cassettes with relevance to pathogenicity and virulence and

had made its way into the genus *Acinetobacter*, members of which are emerging opportunistic pathogens (Fig. 6).

It also appears as if the MITE-integron complex is resident on a large genomic island and that this island is highly mobile, since it has now been detected in three different species of *Acinetobacter*, all resident in the guts of prawns (241). The presence of the MITE-integron within a bacterium found in a food item that is consumed whole, and lightly cooked, suggests a clear pathway for reentry into human hosts. Identical MITE sequences have been detected at the termini of antibiotic resistance integrons in several strains of clinical *Acinetobacter baumannii* and in *Acinetobacter bereziniae* (242, 243), strongly suggesting that this kind of element is readily transferrable between *Acinetobacter* species. In the case of the *Acinetobacter* MITE-integron, mobility may be conferred by the MITEs themselves or by residency on a genomic island. In *Enterobacter cloacae*, integron mobility is associated with a different, but functionally related, MITE (33).

Consequently, simultaneous pollution with integrons and selective agents has the potential impose new selective forces on environmental microorganisms (244). These secondary, unanticipated effects of the antibiotic revolution will precipitate evolutionary change among microorganisms across the globe and have potentially adverse consequences for human welfare (153, 245).

Integrons as Tools for Biotechnology

Integrons have some significant advantages as a platform for biotechnological applications. They have all the machinery for acquisition, rearrangement, and expression of exogenous genes, in a tractable *in vivo* system. Natural integron integrase activity can be used to recover functional gene cassettes into a plasmid background for further downstream manipulation (246). Synthetic and natural gene cassettes can readily be introduced into cells via natural transformation. This potentially allows any gene to be incorporated into an integron (247). Further, marker cassettes, such as gene cassettes encoding green fluorescent protein, could be used to naturally transform environmental bacteria with active integron recombination systems. This would allow chromosomal cassette arrays to be easily recovered from environmental samples and would detect arrays in yet-to-be cultured organisms.

Chromosomal cassette arrays are a vast resource for discovery of novel proteins (70, 97, 98) and for the discovery of protein folds that might comprise building blocks for the flexible assembly of new proteins (100). Such cassette arrays have already been subjected to natural selection in the environment where they are found and so are likely to encode proteins of adaptive relevance (16, 98, 112). Consequently, the search for proteins with specific properties and/or activities might be made more efficient by searching natural environments that match the desired conditions under which the protein needs to operate and then screening for the appropriate gene cassettes. These cassettes need not be members of any recognized protein family.

Once candidate genes have been assembled into a cassette array, the natural activity of integrons could be used to generate diverse arrangements of all the component genes. Selection for optimized activity would then recover the variant cassette array with an optimum content and order of the component gene cassettes. Proof of principle has already been demonstrated via optimization of the tryptophan pathway using synthetic gene cassettes randomly rearranged via integron integrase activity (248, 249). Thus, integron platforms could be used to generate new biochemical pathways for bioremediation or biosynthesis through integron-mediated operon engineering (20, 98).

Increasing Bacterial Evolvability

The use of antimicrobial compounds has driven the fixation of ever more complex DNA elements containing integrons, resistance genes, transposons, and other mobile DNAs within all human-dominated ecosystems. These xenogenetic DNA elements are released back into the environment, simultaneously with the antibiotics, disinfectants, and heavy metals that originally drove their selection (175). Wastewaters and effluent then become a giant reaction vessel for recombination and rearrangement of resistance determinants (17, 229, 250) and for extensive lateral gene transfer between clinical, commensal, and environmental bacteria (145, 236). There is also considerable scope for coselection. As an example, selection for resistance to disinfectants or heavy metals fixes all the other genes linked on the same mobile element, and the more resistance determinants that are present on an element, the more likely coselection will become (28, 129, 175, 233–235). Aquatic environments are likely to be major foci for complex interactions between integrons, resistance determinants, and mobile DNAs (146, 153, 228, 237), where biofilms in particular are a hot spot for genetic exchanges (17, 58, 251).

Human use of selective agents will continue to drive the assembly of complex mosaic elements that, increasingly, will capture genes conferring virulence, transmissibility, and pathogenicity. However, these same selective agents will also have much broader effects on the whole microbial biosphere and on the general tempo of microbial evolution (153). Genetic diversity in bacteria is generated by mutation, recombination, and lateral gene transfer. The rate for each of these processes is under stabilizing selection, balancing the advantages of genetic innovation against the potential for loss of genomic integrity. Not surprisingly, under stable conditions, genetic change is suppressed. However, under conditions of selection or stress, inherent rates of recombination, mutation, and lateral transfer increase under the SOS response (252, 253).

Continued exposure to variable, subinhibitory levels of selective agents creates a circumstance where lineages with higher rates of genetic change have an advantage (254, 255). Thus, an unintended consequence of the antibiotic revolution might be the fixation of bacterial lineages with inherently higher basal rates of mutation, recombination, and lateral gene transfer (153, 175). Clearly, we need to monitor the environmental effects of bioactive pollutants much more carefully.

CONCLUSIONS

Integrons are remarkable genetic platforms with the ability to acquire, rearrange, and express diverse genes sampled from the microbial pangenome. Their facility for seamless acquisition of adaptive phenotypes brought them to sharp attention when they turned this activity toward disseminating antibiotic resistance among clinical pathogens. Research over the last decade has revealed that integrons are far more than a curious phenomenon of clinical concern. They are an ancient, diverse, and widespread mechanism for generating genomic novelty and triggering adaptive responses in bacteria. Understanding their evolution and biology will inform both clinical practice and our ability to manage natural environments. However, there are still outstanding questions about integron function and biology.

First, there are questions about the size of the resource com-

manded by integrons. How many different integron integrase classes are there, and in which taxa do they occur? How many different gene cassettes are available for acquisition by these integrases, what functions do they encode, and what is their contribution to fitness? What factors regulate the acquisition and rearrangement of gene cassettes within arrays? Finally, how are cassettes generated? The conservation of *attC* sites within chromosomal arrays suggest a mechanism that operates within the host cell, and the compact nature of the cassettes themselves suggests that reverse transcription might be involved. However, for the moment these are just speculations, and no integron-associated reverse transcriptase has ever been described. Certainly, understanding the processes that govern cassette generation, diversity, and dynamics would help our management of antibiotic resistance and provide a powerful platform for biotechnology, where potentially any gene could be accessed, manipulated, and expressed using integron activity.

A second set of questions relates to the role of integrons in lateral gene transfer. It is clear that chromosomal integrons can move between genetic locations and between cells. The conservation of breakpoints, for instance in chromosomal class 1 integrons (34), suggests site-specific recombination or transposase activity, but the mechanism(s) involved has not been identified. There are also other unanswered questions about the lateral transfer of integron components. Are there pathways of integron and gene cassette sharing between all taxa, or do cellular and recombination barriers restrict gene cassette exchanges mainly to closely related groups? What are the dynamics of lateral exchange, and how could these rates be controlled? Understanding the processes that regulate integrase activity would aid our ability to control bacterial growth and manage antibiotic resistance. It would also improve the potential of using integrons as platforms for synthetic biology.

A final set of questions deals with human impacts on the microbial world. What is the fate of integrons and gene cassettes released into the environment? What is the fate of the antibiotics, metals, and disinfectants that pollute the same waste streams? Does this copollution significantly affect the background rates of evolution in the whole microbial world, not just the targets of antimicrobial therapies? Can we reduce this potential impact by controlling the release of DNAs and selective agents?

Understanding antibiotic resistance and integron activity at a global scale has important payoffs. There is potential for better health outcomes, better environmental management, and better understanding of the broad sweep of microbial evolution. Whatever approach is taken, it does need to be global, since recent discoveries about integron activity suggest that these versatile elements are potentially capable of sampling and expressing any gene from the microbial biosphere.

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