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Many Class I Integrons Comprise Distinct Stable Structures Occurring in Different Species of *Enterobacteriaceae* Isolated from Widespread Geographic Regions in Europe

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Three sizes of inserted regions of DNA (800, 1,000, and 1,500 bp) were shown to be common among class I integrons in unrelated clinical isolates of *Enterobacteriaceae* from different European hospitals. Sequencing showed that 800-bp inserted regions comprised identical sequences including *aacA4*, that 1,000-bp inserted regions included *aadA*, and that 1,500-bp inserted regions included *dfrI* and *aadA1*, irrespective of host species and geographic origin. In addition promoter sequences were mostly identical for each size class. These data suggest that inserted gene cassettes and promoter regions of integrons are conserved and stable, with resistance genes transferred more often as part of the entire integron structure than as individual gene cassettes.

Integrons are genetic structures capable of integrating or mobilizing individual gene cassettes encoding antibiotic resistance determinants. Since integrons are carried on plasmids and transposons, a strong antibiotic selective pressure can potentially result in the mobilization and dissemination of antibiotic resistance genes (4). Previous studies have demonstrated that these integron structures occur widely among *Enterobacteriaceae* species in European hospitals and are significantly associated with resistance to multiple classes of antibacterial compounds (5, 10).

Integrons possess two essential elements located at the 5′ conserved segment (CS) able to mobilize and insert gene cassettes, namely an *int* gene encoding a site-specific recombinase belonging to the integrase family and its associated primary recombination site, *attI* (3). Captured genes, most commonly encoding antibiotic resistance, are part of discrete mobile cassettes which contain the protein coding region and a recombination site, known as a 59-base element, located at the 3′ end of the gene (8, 9). While four types of integrons, each with different *int* genes, have been identified to date, most integrons found in clinical enterobacterial isolates are class I integrons.

So far more than 40 different antibiotic resistance genes have been shown to be carried within class I integrons, alone or in various combinations, suggesting a high transferability of these genes, which can be integrated, mobilized, or conserved. Inserted genes are expressed primarily via a common promoter, P_1, located in the 5′ CS. Three versions of P_1 are known to exist, with different combinations of −35 and −10 sequences in comparison to consensus sequences. These are TTGACA N_{17}TAAACT (a strong promoter), TGGACAN_{17}TAAGCT (a weak promoter), and TGGACAN_{17}TAAACT (a hybrid promoter) (7, 18). Such sequence changes may be a crude mechanism of control of gene expression. In addition the insertion of three guanosine molecules 119 bases downstream of the promoter P_1 creates a downstream secondary weak promoter, P_2, resulting in a second initiation point of transcription, thus increasing the expression of inserted gene cassettes (3, 7) (Fig. 1). In *Escherichia coli* a strong P_1 and a weak P_1 in combination with P_2 are six and three times more efficient than the *tac* promoter, respectively. Although not yet studied, it is assumed that these promoters behave with a comparable efficiency in other enterobacterial species (7). Genes adjacent to the common promoter P_1 are expressed with the highest efficiency, although promoter variety, plasmid copy number, and the presence of other internal promoters may also affect expression.

Together, the genetic idiosyncrasies of class I integrons suggest that they are fluid genetic elements capable of rapidly mobilizing or capturing resistance genes in response to environmental pressures. However, a previous study of ours, using a targeted PCR protocol to amplify inserted gene cassettes adjacent to the 5′ CS, demonstrated that a few distinct size groups of inserted regions of DNA predominate in bacteria isolated from different European hospitals (10). These most commonly comprised inserted DNA regions of 800, 1,000, and 1,500 bp, presumably consisting of single or multiple gene cassettes. In most bacteria only one integron was detected per isolate, although a few contained two or even three regions of inserted DNA of different sizes, including sizes larger or smaller than the predominant size classes, a fact that is suggestive of multiple integron carriage. These predominantly common sizes suggested at least that inter- and intraspecific gene transfers occur often, by the mobilization of a complete integron structure rather than individual resistance gene cassettes. We hypothesized that integron structures, at least in the predominant size classes, were possibly more stable than had been suggested previously.

In order to test this hypothesis we sequenced the 5′ CS promoter regions and gene cassettes, inserted between the 5′ CS and a semiconserved downstream 3′ region, of integrons of 21 enterobacterial organisms harboring the three most common size groups of inserted DNA regions found. Oligonucleotides and PCR conditions used to amplify integron structures for sequence analyses were those described previously (6). Sequencing reactions on amplicons were performed on both strands with PCR cycle sequencing kits (Perkin-Elmer, Gouda, The Netherlands) according to the manufacturer's instructions. All organisms studied were recent clinical isolates from blood, shown to be unrelated by virtue of random amplified polymorphic DNA typing by a standard procedure (10, 15).

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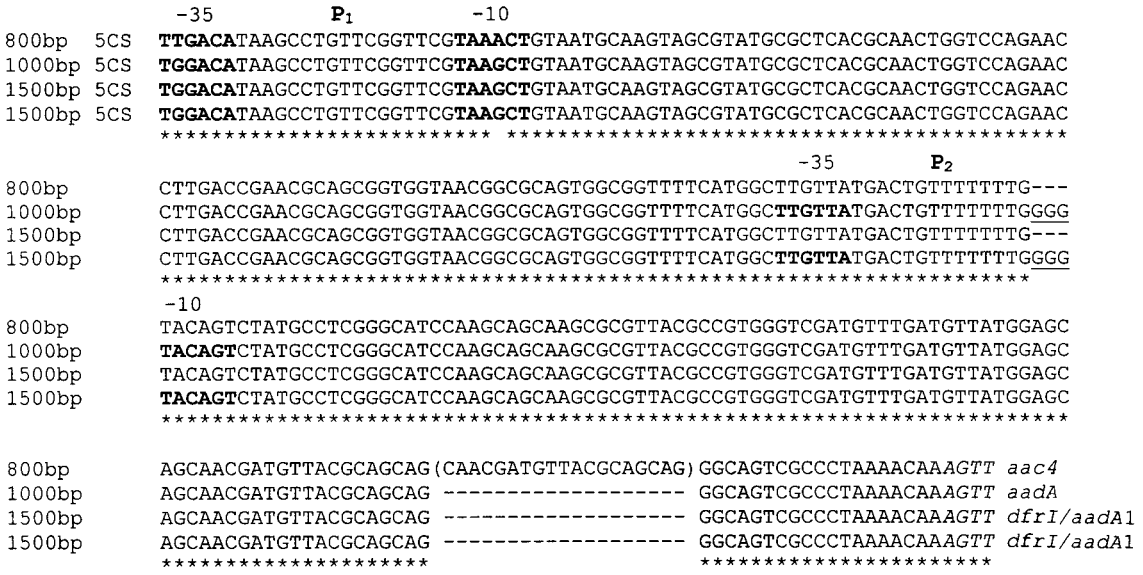

FIG. 1. The 5′ CS regions of class I integrons showing four different combinations of promoter sequences and inserted gene cassettes found among isolates studied. Promoter sequences are shown in bold type and are marked as P_1 or P_2. Isolates with an 800-bp region inserted carry a strong P_1 promoter and *aacA4* gene and a unique 19-bp inserted sequence shown in parentheses. Isolates with 1,000-bp inserted regions possess a weak P_1 promoter plus a weak P_2 promoter made active by the insertion of three guanosine residues (underlined) and an *aadA* inserted gene cassette. Two groups of isolates with 1,500-bp inserted regions were identified; both have *dfrI* and *aadA* as inserted gene cassettes, but one possesses a weak P_1 promoter and the other possesses a weak P_1 promoter plus a weak P_2 promoter made active by the insertion of three guanosine residues (underlined). The unique *attI* site AGTT followed by the inserted resistance gene in each sequence is shown in italics.

Isolates originated in 10 different locations (Düsseldorf and Freiburg, Germany; Warsaw and Lodz, Poland; Seville and Madrid, Spain; Paris, France; Genoa, Italy; Liege, Belgium; and Bristol, United Kingdom) and belonged to six different species (*E. coli*, *Klebsiella oxytoca*, *Enterobacter cloacae*, *Enterobacter aerogenes*, *Proteus mirabilis*, and *Serratia marcescens*).

Sequencing revealed that all isolates containing 800-bp inserted regions (four isolates) comprised *aacA4* alone (encoding resistance to tobramycin and amikacin), those with 1,000-bp inserted regions (nine isolates) all comprised *aadA* (encoding resistance to spectinomycin-streptomycin), and those with 1,500-bp inserted regions (eight isolates) comprised *dfrI* and *aadA* (encoding resistance to trimethoprim and spectinomycin-streptomycin). All isolates with the 800-bp inserted region carried *aacA4*, downstream of a so-called strong P_1 promoter, and a unique 19-bp region in the attachment sequence of the 5′ CS (Fig. 1). Two of these isolates, both from Liege, also carried two additional detectable inserted regions of unique sizes (>2,000 bp) which were not studied. All *aadA* genes contained within 1,000-bp inserted regions were downstream of weak P_1 promoters, with the exception of one *E. cloacae* isolate from Italy. In this isolate the *aadA* gene was also present but was linked to a 5′ CS region different from the 5′ CS region upstream of the *aacA4* gene characteristic of the 800-bp insert, in that no 19-bp repeat sequence was present. This is suggestive of an identical integron structure mutated within the promoter region, changing the P_1 promoter type from weak to strong. Four of the eight 1,500-bp inserted regions comprising *dfrI* and *aadA* genes possessed weak P_1 promoters, with all others having a weak P_1 promoter plus a weak P_2 promoter downstream (Fig. 1). Thus, in every isolate we studied, the nucleic acid sequences of the promoter regions and inserted resistance gene(s) within each inserted region size class were identical, irrespective of the geographic source and species (except for those with 1,500-bp inserted regions which formed two distinct promoter-type groups, each with identical inserted gene sequences).

If the mobilization and subsequent integration of resistance gene cassettes were common occurrences in response to changing antibiotic selective pressures we would perhaps expect to find considerable variation in the combinations of inserted gene cassettes and promoter types. However, our results refute this hypothesis, as we show that the most commonly found combinations of resistance genes and promoter sequences are highly conserved in different species isolated in different European hospitals (Table 1). A similar conservation of promoter and cassette combination was previously reported among dihydrofolate reductase genes carried on unrelated plasmids of diverse origins (20, 21). Rather than suggesting that integrons and the inserted genes they contain are highly mobile and changeable structures recently evolved in the hospital environment, this suggests that they are evolutionarily older and more stable, perhaps originating from a common source. A recent report demonstrated that *aadA* is commonly carried in integrons in *Enterobacteriaceae* organisms in a London hospital, despite the limited use of streptomycin (2). Although many different resistance genes have been reportedly carried within integrons and the role of the integrase in the insertion and excision of gene cassettes has been clearly shown (14), the common occurrence of conserved integron structures in different species strongly suggests that the intra- or interspecific transfer of the entire integron (presumably via plasmids or transposons) is a more frequent event than single gene mobilization or integration via the integrase. However, our studies do not preclude the possibility that other integrons harbor more mobile inserted gene cassette combinations.

Furthermore, we attempted to induce changes within the integron structure in vitro. Clinical isolates representative of each of the species studied that harbored integrons with 800-bp inserted regions (*aadA* plus a weak P_1 promoter and a P_2

TABLE 1. Combinations of inserted resistance genes and promoter types (6), in relation to the size of the inserted region of DNA, found in integrons detected in unrelated clinical enterobacterial isolates from geographically diverse European hospitals

Inserted gene(s)	Promoter type(s)	Species	Geographic origin
800-bp inserted region (*aacA4*)	P_1 strong	*E. coli*	Paris
	P_1 strong	*E. coli*	Liege
	P_1 strong	*E. cloacae*	Warsaw
	P_1 strong	*E. aerogenes*	Liege
1,000-bp inserted region (*aadA*)	P_1 weak + P_2 weak	*E. coli*	Freiburg
	P_1 weak + P_2 weak	*K. oxytoca*	Düsseldorf
	P_1 weak + P_2 weak	*E. cloacae*	Madrid
	P_1 weak + P_2 weak	*E. coli*	Genoa
	P_1 weak + P_2 weak	*P. mirabilis*	Seville
	P_1 weak + P_2 weak	*P. mirabilis*	Paris
	P_1 weak + P_2 weak	*E. coli*	Paris
	P_1 weak + P_2 weak	*P. mirabilis*	Liege
	P_1 strong	*E. cloacae*	Genoa
1,500-bp inserted region (*dfrI-aadA1*)	P_1 weak	*E. coli*	Lodz
	P_1 weak	*K. oxytoca*	Paris
	P_1 weak	*S. marcescens*	Paris
	P_1 weak	*E. coli*	Lodz
	P_1 weak + P_2 weak	*E. coli*	Seville
	P_1 weak + P_2 weak	*E. coli*	Paris
	P_1 weak + P_2 weak	*E. coli*	Bristol
	P_1 weak + P_2 weak	*E. coli*	Liege

promoter) or 1,500-bp inserted regions (*dfrI-aadA* genes plus a weak P_1 promoter) were passaged in triplicate, in Mueller-Hinton broth or Mueller-Hinton agar plates with doubling dilutions (1 to 1,024 mg/liter) of streptomycin, in order to try to induce changes in the P_1 promoter region (from weak to strong) or resistance gene order (shift of *aadA* adjacent to the P_1 promoter). Despite several attempts, no colonies for which MICs of streptomycin were increased, compared to the parent strain, were obtained, and subsequent sequencing of the amplified integron from randomly selected overnight colonies was not able to detect nucleotide sequence changes in any of the integrons studied.

Recently the finding of class IV integrons has demonstrated that these genetic structures function in bacterial genome evolution (11), probably through the fixed integration of genes which confer selective advantages at secondary sites. This role for antibiotic resistance cassettes has been previously proposed (14) but has not been addressed with sample isolates that are diverse with respect to species and geographic origin as we have done. Our data show a high frequency of particular combinations of antibiotic resistance genes, associated with distinct promoter sequences circulating in European hospitals and seemingly transferred both inter- and intraspecifically, and thus tend to support this theory.

In conclusion, class I integrons containing the most commonly found insert sizes seem not to be fluid genetic elements, frequently sequestering and mobilizing resistance genes. Rather, they seem often to comprise conserved stable structures which are mobilized en masse, often concomitantly with other genetically associated resistance determinants. The decreased susceptibility or resistance to antibiotics provided by integrons and their association with other episomal elements also involved in antimicrobial resistance (10, 12, 13, 22) can explain their widespread occurrence within the nosocomial environment.

Nucleotide sequence accession numbers. Promoter sequences shown in Fig. 1 are identical to those first identified by Stokes and Hall (18), appearing in the EMBL and GenBank databases as accession no. X04555, strong P_1 promoter (1); X12868, weak P_2 promoter (19); and X12618, weak P_1 promoter plus weak P_2 promoter (16, 17).

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