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Integrons and Gene Cassettes in the *Enterobacteriaceae*

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Integrons were detected in 59 of 120 (49%) urinary isolates of *Enterobacteriaceae* by PCR using degenerate primers targeted to conserved regions of class 1, 2, and 3 integrase genes. PCR sequencing analysis of the cassette arrays revealed a predominance of cassettes that confer resistance to the aminoglycosides and trimethoprim.

Dissemination of antibiotic resistance genes by horizontal transfer has led to the rapid emergence of antibiotic resistance among clinical isolates of bacteria (20). The spread of resistance genes is greatly enhanced when they form part of a mobile gene cassette, since this provides for horizontal transfer by several mechanisms. These mechanisms include (i) mobilization of individual cassettes by the integron-encoded integrase (9), (ii) movement when the integron containing the cassette relocates—probably by targeted transposition (6, 10, 19), (iii) dissemination of larger transposons such as Tn*21* carrying integrons (16), and (iv) movement of conjugative plasmids containing integrons among different bacterial species. It is therefore not surprising that many of the antibiotic resistance genes found in clinical isolates of gram-negative microorganisms are part of a gene cassette inserted into an integron (21). The four classes of integron so far identified (classes 1, 2, 3, and 4) are distinguished by their respective integrase (*int*) genes (1, 18, 21). Class 4 is a distinctive class of integrons located in the *Vibrio cholerae* genome and is not known to be associated with antibiotic resistance (18).

Gene cassettes consist of a gene flanked by a recombination site, known as a 59-base element, which is recognized by the integron-encoded site-specific recombinase (IntI). Gene cassettes can exist as free circular molecules (9) and are transcribed only when captured and inserted into an integron, usually at the *attI* recombination site 104 bp upstream of the *intI1* gene (13). New cassettes are continually being discovered, and now over 60 cassettes that confer resistance to a range of antimicrobial agents have been identified (14, 21, 23).

Despite the detailed understanding of the molecular relationship between gene cassettes and integrons (13, 21), there is a paucity of information on how widespread these elements are in a hospital setting. Only a few studies have suggested that integrons are widespread in both animal and human clinical bacterial isolates (3, 15, 17, 22). In this study we have determined the incidence of integrons and their class and characterized the cassette arrays in a collection of random isolates collected from nine clinical settings in Sydney, Australia.

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Clinical isolates. One hundred and twenty urinary isolates identified as belonging to the *Enterobacteriaceae* were studied. The isolates were randomly selected during a six-month period (August 1998 to January 1999) from patients in 46 wards of seven hospitals and two community clinics. These organisms were identified as *Escherichia coli* ($n = 90$), *Proteus* sp. ($n = 13$), *Klebsiella* sp. ($n = 15$), and *Enterobacter* sp. ($n = 2$) using standard biochemical criteria (2). The diversity of the 120 isolates was assessed by calculating Simpson's index, using identification of bacterial genus ($n = 4$) and antibiotic profiles ($n = 52$). The probability of selecting two strains at random from different genera with different antibiotic profiles was calculated to be 94.2%.

Antibiotic resistance profiles. Sensitivity profiles were established by agar diffusion using the calibrated dichotomous sensitivity test (4, 5). Isolates were tested for susceptibility to a panel of 18 antibiotics, representing 11 classes (Table 1). Integrons were strongly associated with multiple-antibiotic-resistant strains, with integron-positive strains demonstrating a greater predilection for antibiotic resistance than integron-negative strains (Table 1). Ninety-seven of 120 (81%) strains were resistant to one or more antibiotic. High levels of resistance were found to antibiotics which have been available therapeutically for a long time including ampicillin (64%), sulfafurazole (59%), streptomycin (48%), tetracycline (39%), and trimethoprim (36%) (Table 1).

Incidence of integrons in 120 isolates of *Enterobacteriaceae*. DNA was extracted from bacteria using standard techniques for the isolation of plasmid DNA, and integrons were detected by PCR with the degenerate primers hep35 (5′ TGCGGGT YAARGATBTKGATTT 3′) and hep36 (5′ CARCACATGC GTRTARAT 3′), which hybridize to conserved regions of integron-encoded integrase genes *intI1*, *intI2*, and *intI3* (23). Fifty-nine of the 120 isolates (49%) were integron positive. The class of the integron was determined by analyzing integrase PCR products by restriction fragment length polymorphism (RFLP) following digestion using either *Rsa*I or *Hin*fI restriction enzyme (Table 2). Restriction analysis revealed that 36% contained a single class 1 integron, 3% contained two class 1 integrons (as determined by the presence of two class 1 cassette PCR products), 6% contained class 1 and 2 integrons, and 4% contained a class 2 integron (Table 3). In total, 58 class 1 integrons and 12 class 2 integrons were identified in 59 of the 120 isolates. No class 3 integrons were detected.

TABLE 1. Association between antibiotic resistance and integrons in 120 isolates of *Enterobacteriaceae*

Antibiotic	Date of discovery[a]	% Resistance of *int*-positive strains[b] (total no. of isolates)	% Resistance of *int*-negative strains[c] (total no. of isolates)	% Resistance of total (total no. of isolates)	Association with integron[d]
Aminoglycosides					
Streptomycin	1944	81.4 (48)	16.4 (10)	48.3 (58)	**<0.0001**
Kanamycin	1957	22.0 (13)	1.6 (1)	11.7 (14)	**0.0004**
Gentamicin	1963	16.9 (10)	3.3 (2)	10.0 (12)	**0.0151**
Tobramycin	1968	16.3 (9)	3.3 (2)	9.2 (11)	**0.0283**
Amikacin	1972	3.4 (2)	1.6 (1)	2.5 (3)	0.6155
Netilmicin	1976	3.4 (2)	1.6 (1)	2.5 (3)	0.6155
Antifolates					
Sulfafurazole	1932	91.5 (54)	27.9 (17)	59.2 (71)	**<0.0001**
Trimethoprim	1961	62.7 (37)	9.8 (6)	35.8 (43)	**<0.0001**
Carbapenem					
Imipenem	1983	0 (0)	0 (0)	0 (0)	
Cephalosporins					
Cephalexin	1969	11.9 (7)	6.6 (4)	9.2 (11)	0.5257
Cefotaxime	1976	11.9 (7)	6.6 (4)	9.2 (11)	0.5555
Cefotetan	1981	1.7 (1)	1.6 (1)	1.7 (2)	1.0000
Penicillin					
Ampicillin	1961	89.8 (53)	39.3 (24)	64.2 (77)	**<0.0001**
Augmentin	1977	8.5 (5)	8.2 (5)	8.3 (10)	0.3910
Other					
Chloramphenicol	1947	30.5 (18)	4.9 (3)	17.5 (21)	**0.0002**
Tetracycline	1948	52.5 (31)	26.2 (16)	39.2 (47)	**0.0048**
Nitrofurantoin	1953	23.7 (14)	19.7 (12)	21.7 (26)	0.6606
Norfloxacin	1980	10.2 (6)	1.6 (1)	5.8 (7)	0.1111

[a] Data obtained from reference 12.
[b] Total number of *int*-positive bacteria, 59.
[c] Total number of *int*-negative bacteria, 61.
[d] Significant values are in bold.

Characterization of cassette arrays. Class 1 integron cassette regions were amplified using hep58 and hep59 as described previously (23). Class 2 integron cassette regions were amplified using hep74 (5′ CGGGATCCCGGACGGCATGC ACGATTTGTA 3′), which binds to *attI2*, and hep51 (5′ GAT GCCATCGCAAGTACGAG 3′), which binds to *orfX*, which is situated downstream of the cassette region within Tn*7* (GenBank accession number AJ002782). Cassette PCR products were restricted with *Rsa*I and *Hin*fI. Two representative products of each distinct RFLP were purified by polyethylene glycol precipitation (11) and sequenced. Analysis of 67 integron cassette regions identified a total of 104 cassettes (Table 3). The cassette regions of three class 1 integrons could not be amplified by PCR, possibly due to the lack of a 3′-conserved segment. Class 1 integrons harbored 11 different cassette arrays (Table 3). The most common types of cassette carried by class 1 integrons were those conferring resistance to streptomycin and spectinomycin. These cassettes represented 53% of all cassettes found and included *aadA1* (39% of cassettes), *aadA2* (9%), and *aadA5* (5%). Streptomycin and spectinomycin are seldom used therapeutically, yet *aadA* gene cassettes remain prevalent within integrons despite the fact that the integron-encoded integrase is capable of excising them (9). Thus, even when antibiotics cease to be used therapeutically, genes encoding resistance to these antibiotics are not necessarily lost. Indeed, if reexposed to the antibiotic, the integron could demonstrate a form of genetic memory whereby cassettes that are found at the 3′ end of the cassette region are repositioned by the integrase, nearer to the promoter, where they are expressed more efficiently (8).

A second aminoglycoside adenylyltransferase gene cassette (*aadB*), encoding resistance to gentamicin, kanamycin, and tobramycin, was detected exclusively in seven *Klebsiella* isolates. Interestingly, five of these seven isolates exhibited extended-spectrum β-lactamase activity, indicating that *aadB* was also associated with this resistance.

dfr cassettes (*dfrA1*, *-A5*, *-A12*, *-A17*, and *-B2*) that confer resistance to trimethoprim represented 27% of cassettes detected (Table 3). It is likely that selection for cassettes carrying *dfr* genes has occurred in this population because trimethoprim is used to treat urinary tract infections, and this could therefore account for their high prevalence. Other individual cassettes identified in class 1 integrons were *orfA* and *ereA2* (resistance to erythromycin).

TABLE 2. RFLP classification of integrase PCR products

PCR product	Enzyme	No. of fragments	Fragment size(s) (bp)
intI1	*Rsa*I	1	491
	*Hin*fI	1	491
intI2	*Rsa*I	2	334, 157
	*Hin*fI	2	300, 191
intI3	*Rsa*I	3	97, 104, 290
	*Hin*fI	2	119, 372

TABLE 3. Characterization of integrons, cassette arrays, and integron-associated antibiotic resistance in 59 *Enterobacteriaceae* isolates

Integrons and cassette arrays[a,b]	Site of collection[c]	Incidence by organism			Total	No. of isolates resistant to antibiotic:[d]							
		E. coli	*Klebsiella* sp.	*Proteus* sp.		AM	CM	GM	KM	SM	SU	TC	TP
Class 1 integrons													
aadA1	H1 (17), H2, H3, H4, H5 (3), C2	23	1	0	24	22	2	1	3	**22**	**23**	7	3
dfrA17-aadA5	H1 (2), H2, H4	4	0	0	4	4	1	0	0	**4**	**4**	2	**4**
dfrA12-aadA2	H1, H2 (2)	3	0	0	3	3	3	3	1	**3**	**2**	3	**3**
dfrA5	H1, H2, H3	2	0	1	3	2	0	0	0	2	**3**	1	**3**
aadB	H1, H2 (2)	0	3	0	3	3	1	**2**	**2**	0	**3**	1	3
dfrA1-aadA1	H1	0	0	1	1	1	1	1	0	**1**	**1**	1	**1**
aadA1-aadA5	H5	1	0	0	1	1	0	0	0	**0**	**1**	0	**1**
drfB2-orfA	H1	1	0	0	1	1	1	0	1	1	**1**	1	**1**
dfrA12	H1	1	0	0	1	1	0	0	0	1	**1**	1	**1**
Class 1 (undetermined)	H1 (2)	1	1	0	2	2	0	0	1	1	**2**	1	2
Two class 1 integrons													
aadB + *aadA2*	H1 (2), H3 (2)	0	4	0	4	4	3	**3**	**3**	**3**	**4**	3	3
Class 1 and 2 integrons													
aadA1 + *drfA1-sat1-aadA1*	H3, H4, H6	3	0	0	3	3	1	0	0	**3**	**3**	3	**3**
dfrA12-aadA2 + *drfA1-sat1-aadA1*	H1, C1	1	0	1	2	1	2	0	0	**2**	**1**	2	**2**
dfrA5-ereA2 + *drfA1-sat1-aadA1*	H1	1	0	0	1	1	1	0	1	**1**	**1**	1	**1**
Class 1 (undetermined) + *drfA1-sat1-aadA1*	H2	0	0	1	1	0	0	0	1	**0**	**1**	1	**1**
Class 2 integron													
drfA1-sat1-aadA1	H1, H3, H4 (2), C1	3	0	2	5	4	2	0	0	**4**	3	4	**5**
Total (% of total)		44 (74.6)	9 (15.2)	6 (10.2)	59 (100)	53 (89.8)	18 (30.5)	10 (16.9)	13 (22.0)	48 (81.3)	54 (91.5)	32 (54.2)	37 (62.7)

[a] Class 2 cassettes are underlined.
[b] For identification of cassettes, accession numbers from references 21 and 24 were used.
[c] Numbers of organisms isolated are shown in parentheses if more than one. H, hospital; C, community clinic.
[d] Bold, antibiotic relevance to integron-associated resistance genes. AM, ampicillin; CM, chloramphenicol; GM, gentamicin; KM, kanamycin; SM, streptomycin; SU, sulfafurazole; TC, tetracycline; TP, trimethoprim.

All 12 class 2 integrons carried the same three cassettes as those found in Tn*7*, namely, *dfrA1*, *sat1*, and *aadA1* (Table 3). This could be explained by the fact that the class 2 integrase gene (*intI2*) contains an early stop codon resulting in a truncated form of the enzyme. The resultant integrase is therefore unable to excise existing cassettes or insert new ones.

Associations between integrons and antibiotic resistance. Integrons were significantly associated with resistance to certain antibiotics including gentamicin, kanamycin, streptomycin, tobramycin, sulfafurazole, trimethoprim, ampicillin, chloramphenicol, and tetracycline (Table 1). However, resistance to only streptomycin, sulfafurazole, and trimethoprim, and to some extent gentamicin, kanamycin, and tobramycin, could be directly related to the presence of resistance genes within the integron (Table 3). The association of the other older antibiotics ampicillin, chloramphenicol, and tetracycline with the presence of an integron is likely to be due to genetic linkage between integrons and conjugative plasmids and transposons.

Conclusion. Gene cassettes conferring resistance to nearly every major class of antibiotics have been identified, with the notable exception of the quinolones. Despite this, the current impact of integrons on resistance in Sydney, Australia, and indeed in other countries (17), appears to be focused towards older antibiotics such as streptomycin, trimethoprim, sulfafurazole, and the early aminoglycosides. However, genes conferring resistance to recently introduced antibiotics are already part of gene cassettes. Examples include bla_{IMP}, which confers resistance to imipenem and broad-spectrum β-lactams (22), and *aacA7*, which confers resistance to modern aminoglycosides such as amikacin and netilmicin (7). These cassettes presumably have not yet received enough selective pressure or had sufficient evolutionary time to encourage their widespread dissemination. However, with the potential of integrons to capture and collect gene cassettes it is likely that they will become more prevalent. Consequently, integrons will continue to threaten the usefulness of antibiotics as therapeutic agents.

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