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Increased Prevalence of Class I Integrons in *Escherichia coli*, *Klebsiella* Species, and *Enterobacter* Species Isolates over a 7-Year Period in a German University Hospital

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The prevalence of integrons in five enterobacterial species was analyzed in 900 blood culture isolates from 1993, 1996, and 1999. Remarkably, the prevalence increased from 4.7% in 1993 to 9.7% in 1996 and finally to 17.4% in 1999 ($P < 0.01$). Within 7 years the combined percentage of P1 strong promoters and P1 weak plus P2 active promoters with high transcription efficacies has increased from 23.1 to 33.3 and finally 60% ($P < 0.05$).

Integrons are genetic structures capable of integrating or mobilizing individual gene cassettes encoding antibiotic resistance determinants (1, 3–6, 9, 12, 16). Previous studies have demonstrated that these integron structures occur widely among *Enterobacteriaceae* in European hospitals and are associated with resistance to multiple classes of antibacterial compounds (4, 7, 10, 11, 14, 15).

Integrons possess two essential elements located at the 5′-conserved segment (CS) that are 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* (3–6). 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. With few exceptions, the gene cassettes in an integron are expressed from a common promoter region located in the 5′-CS of the integron. The promoter region contains two potential promoters called P1 and P2. Four different P1 promoters (a strong, a weak, and two hybrid promoters) and two different P2 promoters (an active and an inactive form) have been described (2, 3, 8). The strengths of the different promoters have been assessed relative to that of the depressed *Escherichia coli tac* promoter (3). The strong version of the P1 promoter is six times more effective than the *tac* promoter, but the *tac* promoter is more efficient than the weak and hybrid P1 promoters. The weak P1 and active P2 promoters, acting together, initiate transcription three times more efficiently than the *tac* promoter (3).

In this study we aimed to analyze potential changes in the prevalence of integrons in five enterobacterial species and to investigate alterations in promoter structure and gene cassette size over a period of 7 years. Therefore, we screened 900 enterobacterial isolates from blood cultures obtained from patients treated at the University Hospital Düsseldorf in 1993, 1996, and 1999. For each year the first 200 isolates of *E. coli* and the first 25 isolates of each of the species *Klebsiella pneumoniae*, *Klebsiella oxytoca*, *Enterobacter aerogenes*, and *Enterobacter cloacae* were analyzed for the presence of class I integrons and the size of the inserted DNA, which gives some indication of the number of inserted genes. We also sequenced the 5′-CS promoter regions of the isolates positive for integrons. The numbers of isolates for the different species reflect the frequency of isolation. Statistical comparisons were performed with Fisher's exact test for proportions, and a Bonferroni-Holm correction was applied to the significance level in cases of multiple comparisons.

Random amplified polymorphic DNA (RAPD) typing of all 300 isolates from each of the three years was used as a rapid screening method to exclude potential clonally identical isolates (10, 13). Of the 300 isolates tested in each of the years 1993, 1996, and 1999, 276, 278, and 287 different RAPD types were distinguished, respectively. Only those isolates considered unrelated were analyzed further in the study.

PCR procedures with various primers to detect the integron structures and to sequence the 5′-CS promoter region have been described previously (10, 11, 15). Primers 5′-CS and 3′-CS were used to identify the presence of an integron and to determine the size of any inserted gene cassette. In addition, primer Int_2F, specific for the 3′ region of the integrase gene (approximately 600 bp upstream from the 5′-CS primer site), was used in combination with primer 3′-CS to show the proximity of inserted gene cassettes to *intI* (10). Two additional primers were specific for the 16S rRNA gene and were used as positive PCR controls ensuring the integrity of all sample DNA used to detect integrons (10). Furthermore, the presence of integron structures as well as of resistance genes in the cassettes of integrons was confirmed by sequencing the PCR products and the contents of the gene cassettes of half of those isolates carrying integron structures, as described previously (11).

Among the 900 isolates tested, 841 different RAPD types were distinguished, of which 90 (16.6%) were shown to carry integron structures. Remarkably, the prevalence of integrons

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TABLE 1. Percentage of integron-positive isolates and sizes of inserted gene cassettes for five different enterobacterial species in 1993, 1996, and 1999

Bacterial species	1993		1996		1999	
	No. of integron-positive/different[a] isolates (%)	Sizes of inserted gene cassettes (bp)	No. of integron-positive/different isolates (%)	Sizes of inserted gene cassettes (bp)	No. of integron-positive/different isolates (%)	Sizes of inserted gene cassettes (bp)
E. coli	5/193 (2.6)	650, 800, 1,500, 1,600, 1,800	12/194 (6.2)	800, 1,000, 1,400, 1,500, 1,600, 1,800, 2,000	20/198 (10.1)	1,000, 1,400, 1,500, 1,600, 1,800, 2,000, 3,000
K. pneumoniae	1/21 (4.8)	750	2/22 (9.1)	750, 1,800	4/23 (17.4)	750, 1,600, 1,800, 3,000
K. oxytoca	3/21 (14.3)	750, 1,600	5/21 (23.8)	750, 1,600	12/23 (52.2)	750, 1,600, 3,000
E. cloacae	2/21 (9.5)	800, 1,000	4/21 (19.0)	800, 1,000, 1,500	8/22 (36.4)	1,000, 1,500
E. aerogenes	2/20 (10.0)	800, 1,000	4/20 (20.0)	800, 1,000, 1,500	6/21 (28.6)	800, 1,000, 1,500
All isolates ($P < 0.01$)	13/276 (4.7)	650, 750, 800, 1,000, 1,500, 1,600, 1,800	27/278 (9.7)	750, 800, 1,000, 1,400, 1,500, 1,600, 1,800, 2,000	50/287 (17.4)	750, 800, 1,000, 1,400, 1,500, 1,600, 1,800, 2,000, 3,000

[a] Of the total number of isolates tested, only those representing different RAPD types were analyzed.

has increased significantly from 13 of 276 (4.7%) in 1993 to 27 of 278 (9.7%) in 1996 and finally to 50 of 287 (17.4%) in 1999 ($P < 0.01$) (Table 1).

K. oxytoca was the species with the highest proportion of integron-positive isolates (20 of 65 [26.8%]). In addition, 6.3% of the *E. coli* isolates (3 of 585), 10.6% (7 of 66) of the *K. pneumoniae* isolates, 21.9% (14 of 64) of the *E. cloacae* isolates, and 19.7% (12 of 61) of the *E. aerogenes* isolates were integron positive (Table 1).

With respect to inserted gene cassettes, sizes of 650 to 1,800 bp were detectable in 1993; by 1999 the cassette sizes had increased to 750 to 3,000 bp. The range of inserted gene cassette sizes detected over the study period varied between 650 bp (found in *E. coli*) and 3,000 bp (in isolates of *E. coli*, *K. oxytoca*, and *K. pneumoniae*). In all species tested, multiple insert sizes were recorded, demonstrating the heterogeneity of inserted sequence sizes. In general, larger inserted gene cassette sizes were detected in 1999. The 3,000-bp inserted gene cassette was detected in *E. coli, K. pneumoniae*, and *K. oxytoca* isolates from Düsseldorf in 1999 for the first time, while the smallest inserted gene cassette of 650 bp could be detected only in *E. coli* isolates from 1993.

By use of the primer sets described previously (10), seven isolates (four *E. coli*, one *K. oxytoca*, one *K. pneumoniae*, and one *E. aerogenes* isolate) were found to possess "empty" integron structures with no inserted gene cassettes. All these isolates originated from 1993; this phenomenon was no longer observed in 1996 and 1999. Thus, increases in integron prevalence and in the sizes of inserted gene cassettes were observed in parallel over the 7 years of the study period.

The different sizes of the gene cassettes inserted between the CS regions of the strains studied demonstrate the variable nature of these structures, presumably reflecting differences in the number and type of inserted gene cassettes. Additionally, many inserted regions of DNA, indistinguishable with respect to size, were detected in isolates from different species or in isolates of the same species shown to be unrelated by genotyping, which is suggestive of horizontal transfer. Recent studies have suggested that 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 (11). However, these studies do not preclude the possibility that other class I integrons harbor more mobile inserted gene cassette combinations.

In addition, the structures of the promoter regions of the integron-positive isolates were analyzed, and a shift in the distribution of different promoters was observed. In 1993, of 13 integron-positive isolates, 8 had P1 weak promoters, 2 had P1 strong promoters, 2 had P1 hybrid promoters, and 1 had a P1 weak promoter plus a P2 active promoter. In 1996 this distribution had changed: of 27 integron-positive isolates, 15 had P1 weak promoters, 6 had P1 strong promoters, 3 had P1 hybrid promoters, and 3 had P1 weak plus P2 active promoters. Finally, in 1999, the following distribution of promoters among the 50 integron-positive isolates could be detected: 17 P1 weak, 23 P1 strong, 3 P1 hybrid, and 7 P1weak plus P2 active. Within 7 years the percentage of promoters with high transcription efficacies, i.e., P1 strong promoters and P1 weak plus P2 active promoters, had increased significantly, from 23.1% (3 of 13) in 1993 to 33.3% (9 of 27) in 1996 and 60% (30 of 50) in 1999 ($P < 0.05$). These data suggest that the genes adjacent to the promoter region were potentially expressed with a higher efficiency in 1999 than in 1993, although besides promoter variety, the plasmid copy number and the presence of other internal promoters may also affect expression.

Compared with the 751 integron-negative isolates, the 90 integron-positive isolates were statistically more often resistant to some β-lactam compounds, e.g., ampicillin (53 versus 37%) ($P < 0.01$), ticarcillin (49 versus 29%) ($P < 0.01$), piperacillin (42 versus 19%) ($P < 0.01$), and trimethoprim-sulfamethoxazole (52 versus 7%) ($P < 0.01$)). This association between integron carriage and decreased susceptibility to certain antibiotics is in line with the findings of previous investigations (10, 15).

While 82% of the integron-positive isolates originated from intensive care units, 10% came from non-intensive-care-unit wards and 8% came from outpatients. The decreased susceptibility or the resistance to antibiotics provided by integrons and their association with other episomal elements also involved in antimicrobial resistance can explain their widespread occurrence in the nosocomial environment with constant or increasing antibiotic pressure. The increased prevalence of integrons observed in a university hospital offers cause for concern and may indirectly assist in our understanding of the

dynamics and molecular basis of multidrug resistance in gram-negative bacteria.

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