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Novel Gene Cassettes and Integrons

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An increase in multiresistant *Enterobacteriaceae* was observed at one of the departments of the University Medical Center Utrecht. Nine different integrons and 17 gene cassettes were found, including the new gene cassette *aadA8*. This cassette was highly related to *aadA3* and *aadA2*. In addition, an unknown promoter sequence was found for two integrons.

In gram-negative bacteria and especially among *Enterobacteriaceae*, class 1 integrons are involved in antibiotic resistance (2, 4, 7, 10, 11). Hall and Collis (3) defined integrons as elements that contain the genetic determinants of the components of a site-specific recombination system that recognizes and captures mobile gene cassettes. Gene cassettes in class I integrons are composed of a single coding sequence and a so-called 59-base element, which is involved in the mobility of the gene cassettes. Most gene cassettes lack a promoter, and expression is dependent on two potential promoters called P1 and P2, which may differ in sequence and strength in different integrons (5). More than 60 gene cassettes have been described (1).

During 1995–1996 an increase of multiresistant *Enterobacteriaceae* was observed at the neurology and neurosurgery department of the University Medical Center Utrecht. Characterization of the increase demonstrated the involvement of class 1 integrons as determined using conserved segment PCR (CS-PCR) (6). Thirty-five of a total of 68 multiresistant *Enterobacteriaceae* (51%) yielded at least one PCR product. At least 11 different PCR products were obtained. DNA sequencing, using the Big Dye Terminator Cycle Sequencing kit (PE Applied Biosystems, Gouda, The Netherlands) and an Applied Biosystems ABI 377 sequencer as described by the manufacturers, showed that two CS-PCR amplification products had no homology with integrons and were considered artifacts. The nine other products analyzed revealed homology with known integrons and gene cassettes (Table 1). The majority of the gene cassettes found have been described before (6, 9) or were presented in GenBank. The *aadA8* gene cassette has not been described before.

The *oxa2* gene has been described before by Stokes et al. as being in an integron carrying two copies of the gene (12). However, our analysis of the published sequences showed that the *oxa2* genes differ by three consecutive base pairs. The first gene in the integron is now called *oxa2a* and the second is called *oxa2b* (R. M. Hall, personal communication). The gene cassette found here was identical to *oxa2a*.

The *ereA2* gene was nearly identical to the gene in GenBank, but the differences with the *ereA1* sequence were larger and

TABLE 1. Characteristics of the CS-PCR products and integrons and GenBank accession numbers of the gene cassettes and highly related gene cassettes

Species	Isolate no.	RFLP type[a]	Size of CS-PCR product (bp)	Gene cassette(s) and order	GenBank accession no.
Klebsiella oxytoca	5	I	1,000	*aadA2*	X68227
		II	1,450	*aadB-catB3*	U13880-U13880
Klebsiella pneumoniae	72	III	2,500	*aacA7-oxa2a-aadA8*	U13880-M95287-AF326210
Klebsiella pneumoniae	139	IV	3,000	*aacA4-aacC1-orf9/orf10-aadA1a*	M55547-L06157-AF326211, U90945,[b] AJ009820[c]-X12870
Escherichia coli	272	V	1,000	*aadA1a*	X12870
Enterobacter aerogenes	302	VI	2,200	*dfrV-ereA2*	X12868-AF326209, AF099140,[d] M11277[e]
Escherichia coli	422	VII	1,550	*dfrIa-aadA1a*	X00926-X12870
Escherichia coli	366	VIII	1,800	*dfrXII-orfF-aadA2*	Z21672-Z21672-X68227
Citrobacter freundii	291	XI	2,200	*orfD-aacA4-catB7*	U13880-M55547-AF227506
Klebsiella pneumoniae	227		1,250		
Klebsiella pneumoniae	366		850		

[a] RFLP, restriction fragment length polymorphism.
[b] U90945 describes the highly related *orfA/B* genes.
[c] AJ009820 describes the highly related *orfX* genes.
[d] AF099140 describes a variant of the *ereA2* gene.
[e] M11277 describes the highly related *ereA1* gene.

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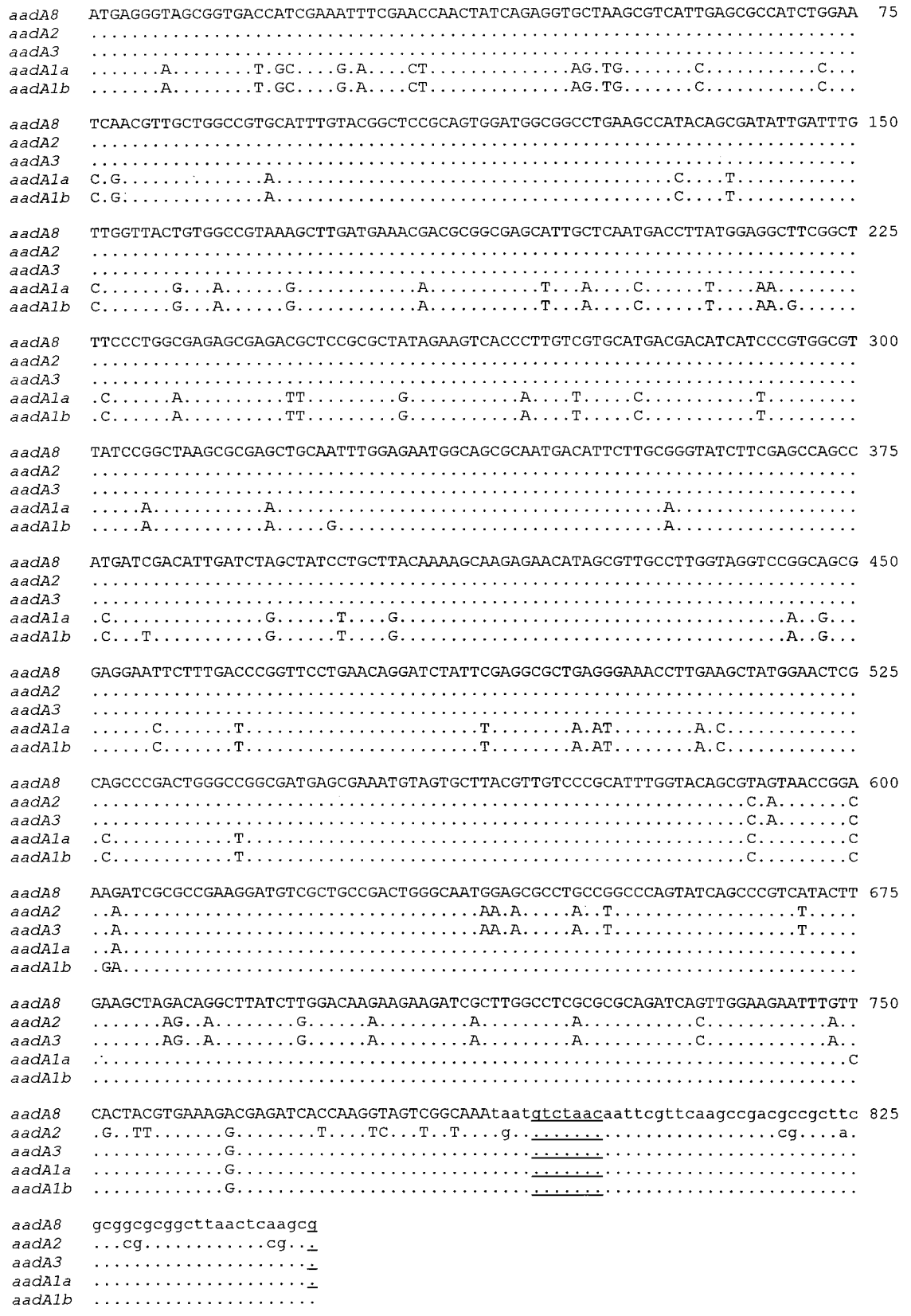

FIG. 1. Alignment of the DNA sequences of *aadA1a, aadA1b, aadA2*, and *aadA3* with the *aadA8* DNA sequence. The homology of the sequences is 93, 91, 96, and 98% when compared with the *aadA8* sequence. Non-codon sequences are in lowercase. The RYYYAAC motif and the G from the GTTRRcY motif delineating the *attC* site are underlined.

Integron	P1	Strength P1 promoter	P2
I	TGGACAtaagcctgttcggttcgTAAGCT	weak	TTGTTAtgactgtttttttggggTACAGT
II	.....g.....	weak	.....---.....
III	.T.....A..	strong	.....---.....
IV	.....	weak	.....---.....
V	.....	weak	.....
VI	.T.....g.....	unknown	.....---.....
	.....	weak	.....---.....
VII	.....	weak	.....---.....
VIII	.....g.....	weak	.....---.....
IX	.T.....g.....	unknown	.....---.....

FIG. 2. Alignment of P1 and P2 sequences. The −35 and −10 boxes are in capital letters, and the spacing between these two boxes is in lower case. The strength of the P1 promoters is indicated. Only the P2 promoters of integron I and V are active.

resulted in significant differences in the EreA1 N terminus, which is 62 amino acids shorter than the EreA2 N terminus. In addition, the next six amino acids were different. A single nucleotide deletion in the sequence of *ereA1* is largely responsible for this large difference. It can be debated whether the difference is either the result of evolution or a sequencing error. Despite the difference in amino acid sequence, putative promoter and ribosome binding sites in front of both coding sequences have been identified, suggesting that the *ereA* gene cassettes can be transcribed from its own promoter sequences, but this does not exclude transcription from the promoters in the 5′-CS region.

The sequences of *orf9*/*10* have been described as part of an integron on pACM1, and a comparison showed a one-nucleotide difference with the sequences labeled *orfA* and *orfB* (8). Seven nucleotide differences are observed in comparison with the *orfX* sequences. The most important difference was a deletion of one nucleotide in the *orfX* sequence. This deletion led to a different and longer C-terminal end for the putative product of the first open reading frame. The other nucleotide differences were observed between the *orf10* and *orfX* sequences, all of them located downstream from the stop codon. A putative GTTRRcY consensus sequence for the end of the *attC* site of *orf9* could not be identified unambiguously. Also, the RYYYAAC consensus sequence for the start of the 59-base element of *orf10* could not be identified unambiguously. Since both open reading frames have been described only together, the possibility exists that *orf9* lacks an *attC* site or that this element is not functional.

The *aadA8* gene cassette was 956 nucleotides long, including a 60-nucleotide 59-base element, and encoded a 262-amino-acid adenylyltransferase. Comparison of the sequence with those of the *aadA1a, aadA1b,* and *aadA2-aadA7* genes showed that it was closely related to *aadA3* and *aadA2* (Fig. 1) and may have arisen by a recombination of those genes. Direct sequence comparison of the *aadA8* gene cassette with its close relatives showed only 20 nucleotide changes when compared to the closely related *aadA3* gene. These differences gave rise to 11 differences in the amino acid sequences specified by the genes. A few additional differences were observed when the *aadA8* gene was compared with the *aadA2* gene, especially in the C-terminal part. Differences with other *aadA* genes were distributed across the sequences. The *attC* site of the *aadA1a* gene had the same *attC* site sequence as both the *aadA8* and *aadA3* genes, whereas the *attC* site of the *aadA1b* gene, which was more closely related to the *aadA1a* gene, differed by five nucleotides from the *attC* site of *aadA8* and *aadA3*.

Analysis of the promoter regions of the nine integrons showed that five different P1 promoter sequences were present, but nucleotide 16, which was either a C or a G, most likely does not influence the activity of the promoter, because it is outside the consensus −35 and −10 sequences. The P1 sequence for integron VI could not fully be resolved, because two variants were present. Part of the P1 sequences, which were obtained from a single isolate, had T and G mutations compared to the rest of the P1 sequences, which had a G and a C at these positions, respectively. Seven of the 10 P1 promoter sequences for the integrons described here were weak promoters, and 1 promoter was a strong promoter, whereas 2 promoters have not been described before and their levels of activity are unknown (Fig. 2). The P2 promoter regions were identical among the nine integrons with the exception of the promoters for integron I and V, which were separated by 17 nucleotide sequences instead of 14 nucleotide sequences. Only the first P2 promoter sequences with 17 nucleotide sequences are active (5).

The detection of at least nine different integrons, one new gene cassette, and two unknown integron promoters isolated from multiresistant *Enterobacteriaceae* from a single specialty (neurology and neurosurgery) at our hospital demonstrates once more the wide distribution of these genetic elements and their potential to contribute to antibiotic resistance.

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