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Recombination between the *dfrA12*-orfF-*aadA2* Cassette Array and an *aadA1* Gene Cassette Creates a Hybrid Cassette, *aadA8b*

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Homologous recombination between closely related gene cassettes, such as *aadA1* and *aadA2*, which are 89% identical, can create hybrid cassettes and hybrids of existing cassette arrays. A new cassette array, *dfrA12*-orfF-*aadA8b*, which was created by such a recombination event occurring within the *aadA2* cassette in the *dfrA12*-orfF-*aadA2* array, has been identified.

Gene cassettes are a major source of the resistance genes found in clinical, commensal, and environmental isolates of bacteria that are resistant to antibiotics. Most commonly, they are found in association with class 1 or class 2 integrons (3, 13). One growing group of gene cassettes encodes aminoglycoside (3′) (9) adenylyltransferases that confer resistance to both streptomycin and spectinomycin (9). The genes and the cassettes, which are named after the genes, are designated *aadA* with an Arabic numeral to distinguish distinct genes, namely, those that differ by at least 2% in both the DNA and protein sequences. Two of these cassettes, *aadA1* and *aadA2*, were present in two of the earliest-known plasmids that confer resistance to multiple antibiotics, namely, NR1, also called R100 (7), and pSa (1, 14), and remain very common in modern-day isolates. Though they have both been found in various contexts, they recur in a few specific cassette arrays, e.g., *aadA1* or *aadA2* alone, *oxa1-aadA1*, *dfrA1-aadA1*, and *dfrA12*-orfF-*aadA2*, which appear to have become globally disseminated. These two cassettes have now been sequenced many times, leading to the identification of several variant sequences for each of them, each containing one or a few single base changes (Fig. 1; also Fig. 3 in reference 9). The high level of similarity between the *aadA1* and *aadA2* cassette sequences (89.3% DNA identity; cassette length, 856 bp) means that they share many stretches of sequence identity that allow homologous recombination between them to occur. Several hybrids between *aadA1* and *aadA2* that presumably arose by homologous recombination have already been reported (Table 1). By combining existing knowledge of variant sequences and of cassette arrays, it is potentially possible to track the movement of specific sets of gene cassettes within bacterial populations and identify events that have been involved in their creation and dissemination.

We used primers located in the 5′- and 3′-conserved segments (CS) of class 1 integrons (HS458, 5′-GTTTGATGT TATGGAGCAGCAACG-3′, and HS459, 5′-GCAAAAAGG CAGCAATTATGAGCC-3′ [5]) to screen DNA isolated from mixed bacterial samples recovered from the feces of human volunteers with no recent exposure to antibiotics. Mixed cultures were grown in L broth at 37°C under aerobic conditions, and the organisms recovered were predominantly *Escherichia coli*. DNA was recovered using alkaline lysis, and PCR conditions were as follows: denaturation at 94°C for 3.0 min; 35 cycles at 94°C for 30 s, 65°C for 1.0 min, and 72°C for 1.5 min or 4.0 min in the final cycle. A significant proportion of the samples screened yielded an amplicon of 2.2 kb corresponding to a cassette array of 1.7 to 1.8 kb when corrected for the amplified portions of the 5′-CS and 3′-CS (465 bp). Partial sequencing of seven of the PCR products revealed that the cassette array was either *dfrA12*-orfF-*aadA2* (in one case) or a derivative in which the end of the *aadA2* cassette is replaced by the corresponding part of the *aadA1* cassette (in six cases) (Fig. 2). The 2.2-kb amplicon from one of the latter samples was cloned into pGEM-T Easy (Promega) by following the manufacturer's instructions, with selection on LB plates containing streptomycin (25 μg ml^{-1}) and trimethoprim (50 μg ml^{-1}), and the resultant plasmid (pMAQ697) also conferred resistance to spectinomycin (25 μg ml^{-1}). The insert was sequenced using procedures described previously (11). The crossover in the hybrid *aadA2*/*aadA1* cassette was located between positions 602 and 647 in the cassette (numbered from the conserved TT at beginning of the cassette) by comparison with the reference *aadA1* (GenBank accession no. X12870) and *aadA2* (X68227) cassette sequences. A similar hybrid with the same crossover position has recently been reported as *aadA8* (AY139603 [15]). However, the original *aadA8* cassette (AF326210 [12]), which is also an *aadA2/1* hybrid, has a different crossover position, between 550 and 600 (Table 1). As the three sequences exhibit high levels (>98%) of DNA and protein identity, we have named the *aadA2/1* hybrid cassettes with crossover between positions 602 and 647 *aadA8b* (Table 1) to indicate the difference in the crossover position.

To examine if the recombination event could have occurred between the *dfrA12*-orfF-*aadA2* cassette array in one integron and an *aadA1* cassette located in a second integron, the sequences were examined in detail. The *dfrA12*-orfF-*aadA2* cassette array was first isolated from an *E. coli* strain from Finland (4), but the sequence of the *aadA2* cassette was not completed

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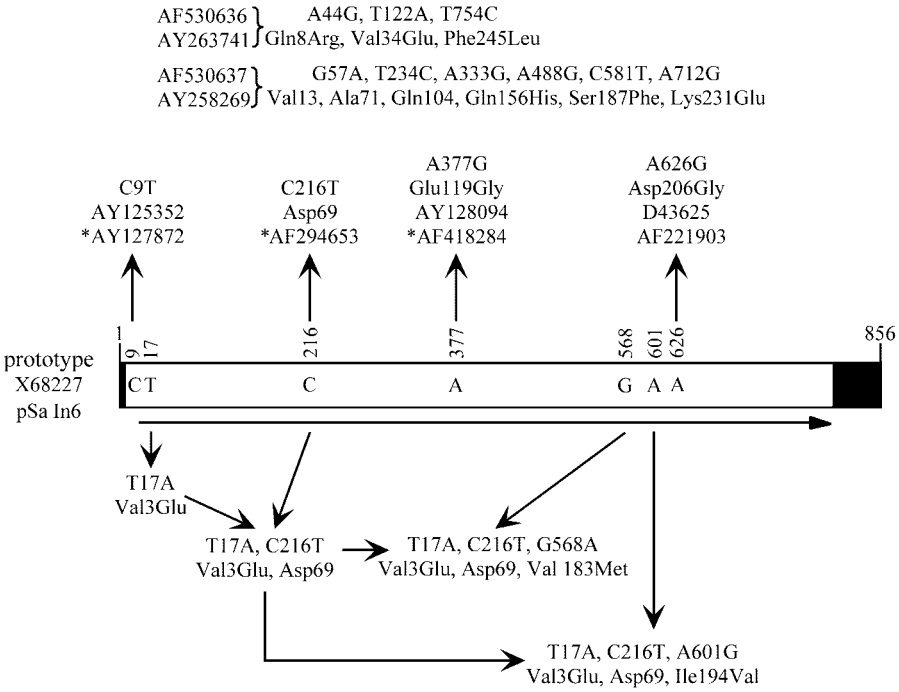

FIG. 1. Variants of the *aadA2* gene cassette. The linear form of the 856-bp *aadA2* cassette is shown, with the 59-base element (59-be) represented by a filled box and an arrow below indicating the extent of the *aadA2* gene. The bases found in the prototype *aadA2* sequence (GenBank accession no. X68227) at positions that can vary are shown in the box, with their positions in the cassette sequence given above. Position 1 is the first T residue of the 1R site (GTTAGAC) of the 59-be. For simplicity, only changes found in at least two of the available *aadA2* sequences are included and variations in the 59-be are not shown. Variant sequences are grouped according to base changes, with any corresponding amino acid changes indicated. Variants shown below the box are found in the *dfrA12*-orfF-*aadA2* cassette array (accession numbers in Table 2). Variants found in other arrays are listed above the box, with accession numbers shown, except for T17A C216T variants found in GenBank accession no. AY263740, *AF458082, *AF486817, and *AY681136 (an asterisk indicates that the sequence contains additional unique changes). Seventeen further sequences are identical to the prototype, and seven more have additional differences found only in these entries.

TABLE 1. Hybrids of *aadA1* and *aadA2* gene cassettes

GenBank accession no.	Name[a]	Original name	Order	Crossover position[b]	Change(s) from standard cassette[c]		Cassette array	Species	Reference or source
					aadA2[d]	*aadA1*[d]			
AF047479	*aadA3*	*aadA3*	*2/1*	758–760		C759T	*aadA3-aacA1*/orfG-orfsH to M-*catB3*		
AF326210[e]	*aadA8*	*aadA8*	*2/1*	550–600		C759T + 4[f]	Unknown	*Klebsiella pneumoniae*	12
AY139603	*aadA8b*	*aadA8*	*2/1*	602–647	A402G[g]	C759T	*aadA8b*	Uncultured	15
AY852272	*aadA8b*		*2/1*	602–647	T17A C216T		*dfrA12*-orfF-*aadA8b*	Mixed culture	This work
AY232671	*aadA1*	*aadA1*	*2/1*	88–101	T17A	C759T	*aadA2*/*1-blaP1*	*Pasteurella multocida*	16
AY171244[e]	*aadA21*	*aadA21*	*2/1*	148–159		GC634-635CG[g]	*aadA21*	*Salmonella enterica* serovar Typhimurium	2
AY550883	*aadA21*	*aadA22*	*2/1*	148–159		G790A[g]	*aadA21*	*Salmonella enterica* serovar Choleraesuis	
AJ809407	*aadA21*	*aadA23*	*2/1*	148–159		G444A[g]	*aadA21*	*Salmonella enterica* serovar Agona	
AB189176	*aadA21*	*aadA23b*	*2/1*	148–159		A529G[g] G790A[g]	*aadA21*	*Escherichia coli*	
AY665771[h]	*aadA12*	*aadA12*	*2/1/2*	148–159 550–600			Unknown	*Escherichia coli*	
AB107663	*aadA1*	*aadA1*	*1/2*	783–786		G732C C759T	*aadA1/2*	*Vibrio cholerae*	

[a] Cassettes with high identity (>98%) to *aadA1* or *aadA21* are named *aadA1* or *aadA21*, respectively.
[b] For boundary *x–y*, the last base identifiable as matching only the "front" cassette is $x - 1$, and the first base identifiable as matching only the "back" cassette is $y + 1$.
[c] Unless otherwise indicated, these differences are found in a number of other variants of the relevant cassette.
[d] The standard cassettes used for comparison are found in GenBank accession no. X12870 for *aadA1*, variants of which commonly have C759T, and X68227 for *aadA2*.
[e] The first 9 nucleotides of the cassette sequence are missing from AF326210, and the last 28 bp of the cassette sequence are missing from AY171244.
[f] The four additional changes are C609A, A612G, G773A (also found in the *aadA1* cassette variant in AY139597), and the unique change C599T.
[g] The changes indicated have not been seen in any other variant of the relevant cassette.
[h] The sequence of the *aadA12* gene only is present in AY665771.

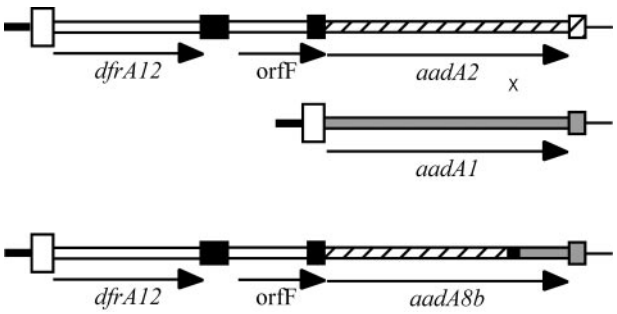

FIG. 2. Schematic of the original and hybrid cassette arrays. The *aadA8b* cassette has arisen by homologous recombination between the *aadA2* cassette (hatched) in a *dfrA12*-orfF-*aadA2* cassette array (e.g., GenBank accession no. AF284063) and an *aadA1* cassette (shaded) in another integron. Cassettes are represented as narrow open boxes with a larger box at one end representing the 59-base element and are shown to scale. × indicates the position of the crossover. The region (between positions 602 and 647) where recombination took place is indicated by a small filled box. The *attI1* site is shown as a tall open box and defines the end of the 5′-CS (thick black line). The thin black line beyond the last cassette represents the 3′-CS sequence.

(GenBank accession no. Z21672). However, this array has since been found in many bacterial species (Table 2) isolated in many different countries from clinical and animal-associated sources and from wastewater. Examination of the sequences of this array recorded in the relevant GenBank entries revealed that five are identical to one another (reference sequence AF284063) and to the incomplete Z21672 sequence. The remainder differ by only a few single base changes (Table 2; Fig. 1), and some of these variations may represent either sequence errors or errors arising during PCR amplification. This suggests that this array arose on a single occasion and has become globally disseminated but subsequently acquired occasional base substitutions.

There are several variant sequences for the *aadA2* cassette found among those deposited in GenBank (Fig. 1), but *aadA2* in the *dfrA12*-orfF-*aadA2* arrays (Fig. 1) usually differs at two positions (T17A and C216T) from the reference *aadA2* cassette found in In6 from plasmid pSa (X68227). In the *dfrA12*-orfF-*aadA2* and *dfrA12*-orfF-*aadA8b* arrays recovered here, the *aadA2* gene cassette and the *aadA2* portion of the *aadA8b* cassette are identical to the *aadA2* variant found in the standard *dfrA12*-orfF-*aadA2* array (AF284063), indicating that the recombination event is likely to have occurred within this array. Beyond the position of the switch to *aadA1*, the sequence of the *aadA8b* cassette is identical to the prototype for *aadA1* (X12870) and to single *aadA1* cassettes recovered from other samples in our commensal mixed-culture collection. In contrast, the variant of *aadA8b* found as a single cassette in AY139603 (15) differs from the version reported here at three positions in the *aadA2* portion and one in the *aadA1* portion (Table 1), indicating that the two variants are likely to have arisen via independent recombination events occurring within the same span (positions 602 to 647).

It is clear that recombination events involving the *aadA1* and *aadA2* cassettes have occurred on several occasions (Table 1) and thus contribute significantly to the creation of new cassette arrays. However, the fact that the *dfrA12*-orfF-*aadA8b* array was recovered from the fecal flora of a number of different

TABLE 2. *dfrA12*-orfF-*aadA2* cassette arrays

GenBank accession no.	Sequence difference(s) in cassette[a]			Organism	Reference or source
	dfrA12	orfF	*aadA2*		
Z21672[b]			—[c]	*Escherichia coli*	4
AF284063[d]				*Serratia marcescens*	
AB154407				*Escherichia coli*	
AB191048				*Staphylococcus aureus*	
AF550415				*Citrobacter freundii*	
AY748453				*Klebsiella pneumoniae*	
AY852272			—[e]	Mixed culture	This work
AB191047		T178C		*Pseudomonas aeruginosa*	
AF175203	AAAA528-31AAA[f]	GG302-303GGG[f]		*Citrobacter freundii*	
AY522923			G568A	*Aeromonas hydrophila*	
AF188331	GGG101-103GG[g]		G568A	*Shigella flexneri*	
AB196348	G300A G360A	G302T[f]	A601G	*Enterococcus faecalis*	
AY115474	C567G[f]	C50G G75A T133C C140G T248C T267G[f]	A601G	Uncultured	15
AY139605	C567G[f]	C50G G75A T133C C140G T248C T267G[f]	A601G	Uncultured	15
AY551331	T115C C179T		A732G	*Salmonella enterica* serovar Choleraesuis	6
AF335108			T216C[h] CCG826-828Δ[f]	*Escherichia coli*	8
AF180731		GC209-210CG	A34G G642T C826Δ[f] G830Δ[f]	*Klebsiella pneumoniae*	

[a] Unless indicated otherwise, these changes are seen only in GenBank entries included in this table.
[b] Standard sequence for the *dfrA12* and orfF cassettes.
[c] Sequence is only available to position 125 of the *aadA2* cassette.
[d] Standard sequence for the complete *dfrA12*-orfF-*aadA2* array.
[e] The *aadA8b* hybrid cassette is identical to the *aadA2* cassette in AF284063 to position 602 and to the standard *aadA1* cassette in X12870 from positions 647 to 856.
[f] In the 59-base element.
[g] This change disrupts the *dfrA12* reading frame, and there are likely errors elsewhere in this sequence (10).
[h] Found in the prototype *aadA2* sequence in X68227.

individuals indicates that it has already become widely distributed, at least in Australia.

Nucleotide sequence accession number. The sequence of the *dfrA12*-orfF-*aadA8b* PCR amplicon has been deposited in GenBank under accession no. AY852272.

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