

# Class 1 Integron-Associated Tobramycin-Gentamicin Resistance in *Campylobacter jejuni* Isolated from the Broiler Chicken House Environment

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**Using PCR, we screened 105 isolates of poultry-associated *Campylobacter jejuni* for the presence of class 1 integrons. Of those isolates, 21% (22 of 105) possessed the integrase gene, but only 5 isolates produced an amplicon in a 5'-3' conserved sequence PCR directed toward amplification of the resistance cassettes. DNA sequencing demonstrated that all five isolates possessed the aminoglycoside resistance gene, *aacA4*.**

*Campylobacter jejuni* is one of the leading causes of sporadic bacterial enteritis in the United States (6). While most infections are self-limiting, invasive infections and chronic colitis are commonly treated with antibiotics (3). Antibiotic resistance is increasingly a serious problem in some pathogens, and the capability for multidrug resistance is a very real concern. Some authorities regard food animals as the primary source of antibiotic resistance genes present in human food-borne pathogens, whereas others regard imprudent use of antibiotics in humans as the major source of the problem (9). Regardless, it is clear that use of antimicrobials both in animals and in humans can select for resistant bacterial populations.

A genetic element called the integron is potentially a major agent in dissemination of multidrug resistance among gram-negative bacteria. The integron contains an integrase gene and a site-specific integration site where the integrase can link antibiotic resistance gene cassettes in tandem in the integration site when the cassette molecules possess a 59-base element (59-be) (14, 32). Presently there are nine known classes of integrons, based on integrase gene homology (17, 25). Over 60 distinct antibiotic resistance gene cassettes have been characterized within integrons, and as many as 7 of them have been found in a single integron at one time (8, 23). Integrons have a broad distribution among gram-negative fecal bacteria of animal origin, and in fact, many plasmids encoding multiresistance in the *Enterobacteriaceae* carry a transposon of the Tn21 family, which contains a class 1 integron (12, 13). In one study, 60% of *Escherichia coli* isolates from poultry possessed a Tn21-like transposon carrying the *aadA1* streptomycin-spectinomycin resistance gene cassette (2). *Campylobacter* species are commonly found among the intestinal flora of chickens, and Gibreel and Skold characterized trimethoprim resistance gene cassettes in *Campylobacter* that were linked to integrase genes

(10, 11). The purpose of this study was to determine whether class 1 integrons are common mediators of antibiotic resistance in *C. jejuni* associated with poultry, since the microflora of the avian intestinal tract are a rich source of these genetic elements.

*C. jejuni* was cultured from the nipple drinker waterline and from cecal droppings from three sequential flocks of broiler chickens within a commercial broiler house by the method of Ransom and Rose (29). *C. jejuni* was similarly cultured from washes of processed broiler carcasses from two different processing plants. Presumptive colonies were confirmed by phase-contrast microscopy and biochemical tests or serological tests per the manufacturer's recommendation (Integrated Diagnostics Inc., Baltimore, Md.). Isolates were screened for possession of the class 1 integrase gene by PCR using a whole-cell template as previously described (2, 26). Positive results were obtained for 22 of the *C. jejuni* isolates ( $n = 105$ ); 7 of 40 (17.5%) of the isolates cultured from carcasses obtained from the processing plants, 10 of 58 (17.2%) of the isolates cultured from chicken cecal droppings, and 5 of 7 (71.4%) isolates from the waterline (nipple drinkers) in the grow-out house possessed class 1 integrase. Because the chickens were culture positive in the grow-out house after *C. jejuni* was cultured from the waterline, we investigated whether the nipple drinkers were the source of the integrase-containing *C. jejuni* which then colonized the broiler chickens. To test this hypothesis, randomly amplified polymorphic DNA PCR was used to genetically type the isolates, using the method described by Payne et al. (26). Seven distinct randomly amplified polymorphic DNA patterns resulted; however, none of the integrase-containing strains from the waterline were present among the strains isolated from chickens. In fact, class 1 integrase was possessed by three different genetic types isolated from the chickens and two genetic types isolated from the waterline in the flock house.

Isolates containing class 1 integrases were assayed for the presence of contiguous resistance gene cassettes, using 5'-3' conserved sequence (CS) PCR with primers targeted to the 5'

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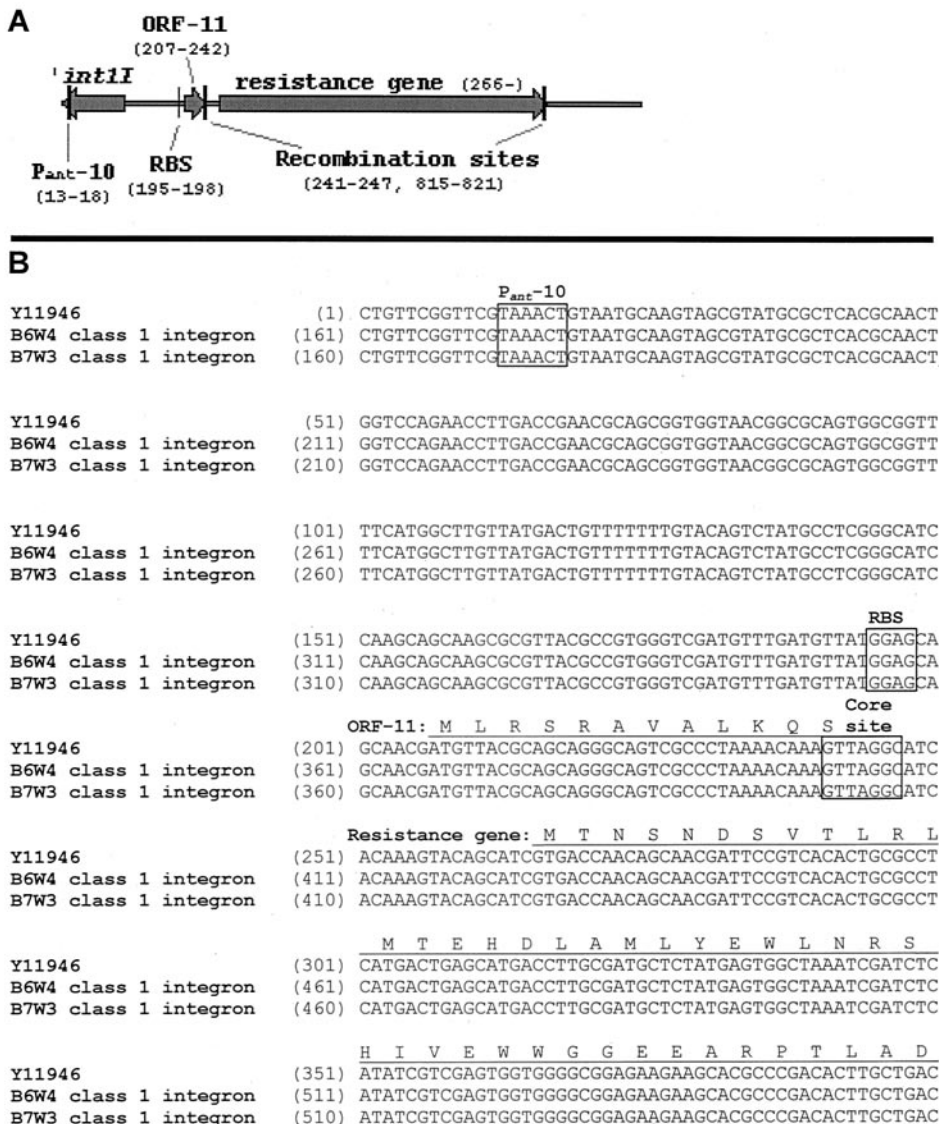


FIG. 1. Comparison of the sequence of *Campylobacter* integron-associated *aacA4* to the sequence submitted to GenBank under accession number Y11946. Panel A shows the schematic annotation of Y11946, which is based on the findings of Hanau-Bercot et al. (16), who demonstrated that expression of the *aacA4* was dependent upon the ribosome binding site (RBS) preceding ORF-11. Panel B shows the homology among the *Campylobacter* integron sequences and Y11946. The locations of the -10 sequence of the P<sub>ant</sub> promoter (7), the ribosome binding site, and the recombination core site of the 59-be (15) are marked with boxes. The derived translation of the ORF-11 and N terminus of AAC(6')-Ib<sub>7</sub> are shown underlined above their respective DNA sequences.

and 3' conserved regions of the class 1 integron sequences (21). When integrase-positive isolates were assayed, only the *C. jejuni* strains isolated from the waterline produced PCR amplicons visible on agarose gels. All five of the waterline isolates produced a 900-bp amplicon, suggesting that they possessed a single cassette in the integron. Amplicons from two waterline isolates were gel purified and DNA sequenced at the campus core facility, using an ABI 9600 automated sequencer. Analysis of the DNA sequences, using the online BLAST algorithm at the National Center for Biotechnology Information web server (www.ncbi.nlm.nih.gov), revealed that they were nearly identical to the nucleotide sequence for the *aacA4* gene. To determine the identity of upstream sequences which could influence expression of the cassette, PCR was performed using primers

int1PS (5'-GAA CAG CAA GGC CGC CAA T-3') and *aacA4*R (5'-TTC TTC TCC GCC CCA CCA CT-3'), which were designed to amplify an area spanning the 3' region of the integrase gene and the 5' region of the *aacA4* gene which would include the putative cassette promoter(s) and 59-be. The primers were based on the sequence submitted to GenBank under accession number AY06966, which also contained *aacA4* as the first cassette in the integron and was predicted to produce a 538-bp product. PCR was done using a 10-μl reaction mixture which consisted of 0.2 mM deoxynucleoside triphosphates, 2.0 mM MgCl<sub>2</sub>, 1× PCR buffer (50 mM Tris [pH 7.4]), bovine serum albumin (0.25 mg/ml), 50 pmol (each) of forward and reverse PCR primers, and 0.5 U of *Taq* DNA polymerase (Boehringer Mannheim, Indianapolis, Ind.). The

AAA25688 [aac(6')II]	(13)	MTErDLpMLhdwLNRpHIVEWwGGdEeRPTLdeVleHylPramaeeSVTP
AF162771_1 [aac(6')IIc]	(13)	MaEnDLpMLhaWLNRPHIVEWwGGedERPTLdeVleHysPeVLakqAvvP
AY029772 [aac(6')Ib]	(1)	MTEHDLAMLYEwLNRSHIVEWwGGEEARPTLADVQEQYLPSVLAQESVTP
Y11946 [aac(6')Ib <sub>7</sub> ]	(13)	MTEHDLAMLYEwLNRSHIVEWwGGEEARPTLADVQEQYLPSVLAQESVTP
B6W4 <i>aacA4</i>	(13)	MTEHDLAMLYEwLNRSHIVEWwGGEEARPTLADVQEQYLPSVLAQESVTP
B7W3 <i>aacA4</i>	(13)	MTEHDLAMLYEwLNRSHIVEWwGGEEARPTLADVQEQYLPSVLAQESVTP
Gen <sup>R</sup> >Ami <sup>R</sup>		
AAA25688 [aac(6')II]	(63)	YIAMLgeEPIGYAQSYVALGSGDGWwEdETDPGVRGIDQSLADptQLnKG
AF162771_1 [aac(6')IIc]	(63)	YIAMLddePIGYAQSYVALGSGDGWwEdETDPGVRGIDQSLANpsQLnKG
AY029772 [aac(6')Ib]	(51)	YIAMLNGEPIGYAQSYVALGSGDGWwEEETDPGVRGIDQSLANASQLGKG
Y11946 [aac(6')Ib <sub>7</sub> ]	(63)	YIAMLNGEPIGYAQSYVALGSGDGWwEEETDPGVRGIDQSLANASQLGKG
B6W4 <i>aacA4</i>	(63)	YIAMLNGEPIGYAQSYVALGSGDGWwEEETDPGVRGIDQSLANASQLGKG
B7W3 <i>aacA4</i>	(63)	YIAMLNGEPIGYAQSYVALGSGDGWwEEETDPGVRGIDQSLANASQLGKG
AAA25688 [aac(6')II]	(113)	LGTrLVraLVELLFsDPtVTKIQTDPtPnNhRAIRCYEKAGFvRekiitT
AF162771_1 [aac(6')IIc]	(113)	LGTKLVRsLVELLFsDPaVTKIQTDPSPSNhRAIRCYEKAGFvqekniIT
AY029772 [aac(6')Ib]	(101)	LGTKLVRALVELLFNDPEVTKIQTDPSPSNLRAIRCYEKAGFERQGTvTT
Y11946 [aac(6')Ib <sub>7</sub> ]	(113)	LGTKLVRALVELLFNDPEVTKIQTDPSPSNLRAIRCYEKAGFERQGTvTT
B6W4 <i>aacA4</i>	(113)	LGTKLVRALVELLFNDPEVTKIQTDPSPSNLRAIRCYEKAGFERQGTvTT
B7W3 <i>aacA4</i>	(113)	LGTKLVRALVELLFNDPEVTKIQTDPSPSNLRAIRCYEKAGFERQGTvTT
AAA25688 [aac(6')II]	(163)	PDGPAVYMVQTRQAFERkRgVA
AF162771_1 [aac(6')IIc]	(163)	PDGPAVYMVQTRQAFESlRtvq
AY029772 [aac(6')Ib]	(151)	PDGPAVYMVQTRQAFERTRsvA
Y11946 [aac(6')Ib <sub>7</sub> ]	(163)	PDGPAVYMVQTRQAFERTRSDA
B6W4 <i>aacA4</i>	(163)	PDGPAVYMVQTRQAFERTRSDA
B7W3 <i>aacA4</i>	(163)	PDGPAVYMVQTRQAFERTRSDA

FIG. 2. Comparison of *Campylobacter aacA4*-derived amino acid sequence to AAC(6')-Ib and -II sequences. *Campylobacter* AAC(6') demonstrates 100% homology to AAC(6')-Ib<sub>7</sub>. The serine residue contributing to gentamicin resistance (Gen<sup>R</sup>>Ami<sup>R</sup>) is marked in bold (30); other dissimilar residues among the AAC(6') enzymes are marked in bold lowercase letters.

program parameters for the Idaho Technology Rapidcycler (Idaho Falls, Idaho) included an incubation at 95°C for 2 min followed by (i) 95°C for 15 s, (ii) 54°C for 15 s, and (iii) 72°C for 30 s for 40 cycles. The PCR product of approximately 550 bp was gel purified, DNA sequenced, and analyzed as described above. Figure 1 shows the sequence homology among the *Campylobacter* integrons and an integron isolated from an *Enterobacter cloacae* (5, 16).

To determine their identities, the 900-bp amplicons from the other three waterline isolates were gel isolated and used as the template in a PCR-enzyme-linked immunosorbent assay (ELISA) as previously described (24). This consisted of the 5'-3' CS PCR using digoxigenin-labeled nucleotides (Roche Molecular Biochemicals, Indianapolis, Ind.) and a biotinylated *aacA4*-specific probe. The 5'-3' CS PCR was performed as previously described (21), except that the reaction mixture (50 mM Tris [pH 8.3], 3 mM MgCl<sub>2</sub>, 0.25 mg of bovine serum albumin/ml, 0.5% Ficoll) contained 0.1 fM of the 3' biotinylated probe 5'-GGCATCCAAGCAGCAAG-3'. After 30 cycles, the amplicons were denatured by incubation for 1 min at 96°C, with probe annealing for 15 min at 50°C. Probe-amplicon hybrids were captured in streptavidin-coated wells and detected using digoxigenin antibody conjugate as described by the manufacturer (Roche Molecular Biochemicals). *C. jejuni* isolates B6W4 and B7W3 were used as the positive controls; *Salmonella enterica* serotype Typhimurium DT104 was used as the negative control (4). All of the *intI1*<sup>+</sup> waterline isolates contained *aacA4*, as determined by DNA-DNA hybridization in the PCR-ELISA. Four of the *aacA4*-containing water isolates (B4W4, B6W2, B6W4, and B7W3) representing one genetic type were cultured during the fourth, sixth, or seventh week of the 49-day grow-out period of the flock, while isolate

B7W1, representing a unique strain, was cultured only during the seventh week. Nipples on drinkers in the grow-out house were randomly chosen for removal and culture; therefore, these strains were not acquired from repeated cultures of the same nipple drinker. Since the two different strains were isolated from physically separate nipple drinkers, it is likely that they independently acquired the integron from a common source that colonized the biofilms of the waterline in the broiler house. However, 5'-3' CS PCR coupled with PCR-ELISA did not detect the cassette among the community DNAs of water samples from other nipple drinkers in the house.

The aminoglycoside resistance gene, *aacA4*, has been found in integrons from multiple species of bacteria and in class 1 and class 3 integrons (1, 22, 27, 28, 33). *aacA4* encodes an aminoglycoside 6'-N-acetyltransferase [AAC(6')-I or -II] which confers resistance to aminoglycosides containing the purpurosamine ring, including tobramycin, kanamycin, and neomycin (30, 33). Point mutations resulting in a threonine-to-serine substitution (amino acid position 102 in the sequence submitted to GenBank under accession number AAA25688) determine whether the gene also confers resistance to amikacin [AAC(6')-I] or gentamicin [AAC(6')-II] (30). Figure 2 shows that the derived amino acid sequence of the *aacA4* allele from *Campylobacter* demonstrated 100% homology to the AAC(6')-Ib<sub>7</sub> enzyme, which has been shown to confer characteristics resulting in higher kanamycin and tobramycin MICs but only slightly increased resistance to gentamicin (5). To evaluate the phenotype conferred by the AAC(6')-Ib<sub>7</sub> enzyme in *C. jejuni*, four of the isolates containing *aacA4* were assayed for tobramycin and gentamicin resistance, using agar dilution on Mueller-Hinton agar containing 5% washed sheep red blood cells

TABLE 1. Tobramycin and gentamicin MICs for class 1 integron-containing *Campylobacter jejuni* isolates carrying the *aacA4* aminoglycoside resistance gene cassette

Isolate	Source <sup>a</sup>	<i>aacA4</i> <sup>b</sup>	Tobramycin MIC (μg/ml) <sup>c</sup>	Gentamicin MIC (μg/ml) <sup>c</sup>
B5-8	Feces	–	<0.5	<0.5
2A4	Carcass	–	<0.5	1
B6W2	Water	+	>50	>50
B6W4	Water	+	>50	>50
B7W1	Water	+	25	10
B7W3	Water	+	>50	>50

<sup>a</sup> *Campylobacter jejuni* was cultured from nipple drinkers in a broiler house (water), cecal droppings of three successive flocks of chickens in the same broiler house (feces), or carcass washes of broilers from two different flocks at the processing plant (carcass). All isolates in this table possess class 1 integrase, as detected by PCR (2).

<sup>b</sup> Presence or absence of *aacA4* cassette in a class 1 integron as determined by 5'-3' CS PCR (21) coupled with DNA sequencing or DNA-DNA hybridization (24). Templates from isolates B5-8 and 2A4 do not produce amplicons in 5'-3' CS PCR.

<sup>c</sup> Resistance phenotype determined by agar dilution (19).

(19). Table 1 shows that for the *int*<sup>+</sup> *aacA4*<sup>+</sup> strains tested, tobramycin MICs were 25 to 50 μg/ml and gentamicin MICs were 10 to 50 μg/ml. Among the *int*<sup>+</sup> *aacA4* strains tested, MICs were <2 μg/ml. These results strongly suggest that the AAC(6') activity is expressed in these *C. jejuni* strains.

The sequences affecting the expression of some integron cassettes are still being elucidated. Past studies have shown that there are two sets of –35 and –10 sequences, upstream of the cassettes, which are present within the class 1 integrase gene itself (7, 20). The first set comprises the promoter P<sub>ant</sub> and is commonly involved in transcription of the cassettes (7). While most class 1 integrons possess this promoter, point changes in the –35 or –10 sequences affect the level of transcription (7). The P<sub>ant</sub> promoter we detected contained a hybrid version (–35, TGGACA; –10, TAAACT) that has been shown to mediate expression in a manner comparable to that of P<sub>tac</sub> (20). The second set of –35 and –10 sequences comprise a putative P<sub>2</sub> promoter, but the promoter has been shown to initiate transcription only when the spacing is at least 17 bp (31). The P<sub>2</sub> region found in many integrons, including this *Campylobacter* tobramycin resistance integron, contains a 14-bp spacer only between the –35 and –10 sequences and probably does not promote expression. For some integrons it is unclear how resistance is expressed, since one cassette may not contain a recognizable translation initiation region while others demonstrate canonical ribosome binding sites. The integron in this work demonstrated high homology to the sequence submitted to GenBank under accession number Y11946 (Fig. 1), which also does not demonstrate the presence of an apparent ribosome binding site linked to the AAC(6')-Ib<sub>7</sub> open reading frame (ORF) (5). Hanau-Bercot et al. demonstrated that efficient translation of the AAC(6')-Ib<sub>7</sub> in this integron occurred because it was coupled to translation of an 11-amino-acid ORF (ORF-11) immediately upstream (16). Expression in *Campylobacter* presumably occurred by the same mechanism.

Although the chickens in this study were not colonized with the same *Campylobacter jejuni* strains present in the waterline, they were colonized with a variety of genetic types that contained the class 1 integrase gene. If gene exchange occurs readily among members of the intestinal microflora, these in-

tegrons should have captured antibiotic resistance genes from cassette-containing genetic elements transferred to *Campylobacter*. Thus, we expected to find the streptomycin resistance gene *aadA1*, because it is present at a very high incidence in class 1 integrons found among poultry *E. coli* (2). However, this gene was not detected among the integrons present in poultry *C. jejuni*, suggesting either that *Campylobacter* and *E. coli* do not readily undergo gene exchange or that the elements are not stably maintained.

Among the aminoglycoside antibiotics that are approved for poultry production (34, 35, 36), *aacA4* does not confer resistance to streptomycin, although it does confer resistance to gentamicin. Gentamicin is used at the hatchery but not on the broiler farms themselves, and there was no antibiotic selective pressure for this gene in the waterlines of the broiler house, since there was no history of antibiotic usage in the flock house. Since *aacA4* was not found among the class 1 integron-containing isolates cultured from chickens, the presence of this gene may reflect human contamination of the farm environment. Studies by Jacobs-Reitsma et al. indicate that flock depopulation is a major risk factor for flock colonization by *Campylobacter*, presumably as a result of the entrance of farmworkers and equipment into the house (18). In the broiler houses we examined, farmworkers often removed nipple waters for cleaning or replacement between flocks. Contamination with human flora might contribute to the species colonizing the biofilm, ultimately affecting the potential gene pool available for gene transfer.

**Nucleotide sequence accession numbers.** The integron-associated *aacA4* sequences from the waterline isolates B6W4 and B7W3 were submitted to GenBank under accession numbers AF439785 and AF439786, respectively.

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