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Cloning and Expression of Novel Aminoglycoside Phosphotransferase Genes from *Campylobacter* and Their Role in the Resistance to Six Aminoglycosides

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ABSTRACT Nine *aph* genes, including *aph(2″)-Ib*, *aph(2″)-Ic*, *aph(2″)-Ig*, *aph(2″)-If*, *aph(2″)-If1*, *aph(2″)-If3*, *aph(2″)-Ih*, *aac(6′)-Ie–aph(2″)-Ia*, and *aac(6′)-Ie–aph(2″)-If2*, were previously identified in *Campylobacter*. To measure the contribution of these alleles to aminoglycoside resistance, we cloned nine genes into the pBluescript and expressed them in *Escherichia coli* DH5α. The nine *aph* expressed in *E. coli* showed various levels of resistance to gentamicin, kanamycin, and tobramycin. Three genes, *aac(6″)-Ie–aph(2″)-Ia*, *aph2″-If1*, and *aph2″-Ig*, showed increased MICs to amikacin, and five *aph* genes were transferrable.

KEYWORDS *Campylobacter*, gentamicin resistance, NARMS, cloning and expression

The aminoglycoside 2″-phosphotransferase [APH(2″)] family is a major contributor to gentamicin resistance (Gen^r) in *Campylobacter*. Nine variants of *aph* genes were previously identified in *Campylobacter* isolated from human and retail chickens, including seven monofunctional *aph(2″)* genes, *aph(2″)-Ib*, *aph(2″)-Ic*, *aph(2″)-Ig*, *aph(2″)-If*, *aph(2″)-If1*, *aph(2″)-If3*, and *aph(2″)-Ih*, and two bifunctional *aph(2″)* genes, *aac(6′)-Ie/aph(2″)-Ia* and *aac(6′)-Ie–aph(2″)-If2*. Five of them, *aph(2″)-Ig*, *aph(2″)-If1*, *aph(2″)-If3*, *aph(2″)-Ih*, and *aac(6′)-Ie–aph(2″)-If2*, were novel genes (1).

Toth et al. showed that *aph(2″)-If* confers resistance to several aminoglycosides (2). Since the APH(2″) family is genetically diverse and the percentage of amino acid identity between subfamilies can be as low as 25.9% (1–3), it is important to investigate transferability and the attribution of variants of *aph* gene resistance to other aminoglycosides, in addition to gentamicin.

Eleven *Campylobacter* strains, five *C. jejuni* and six *C. coli*, obtained from the National Antimicrobial Resistance Monitoring System (NARMS) program were used in this study (Table 1). All isolates were previously sequenced using the Illumina MiSeq (Illumina, San Diego, CA, USA) (1, 4). The coding sequences of nine *aph* genes were synthesized and cloned in a pBluescript expression vector by GenScript (Piscataway, NJ). For two bifunctional *aph(2″)* genes, only the *aph(2″)-Ia* and *aph(2″)-If2* genes were cloned into the pBluescript vector. The pBluescript::*aph(2″)* recombinant plasmids were transformed into competent *Escherichia coli* DH5α, according to the protocol provided by Thermo Fisher Scientific (Invitrogen, Carlsbad, CA). The clones were grown on LB agar plates containing 50 μg/ml ampicillin and 8 μg/ml gentamicin.

Expression of the cloned *aph* genes in *E. coli* DH5α was first determined by MICs of gentamicin using broth microdilution (CMV3AGNF; Thermo Fisher Scientific, Trek Diagnostics, Cleveland, OH), following standard protocols (5). *E. coli* DH5α carrying the pBluescript vector without the *aph* genes was used as a control. To measure the contribution of different *aph* genes to additional aminoglycoside resistance, agar dilution was performed, and the MICs of six aminoglycosides, including gentamicin,

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TABLE 1 *Campylobacter* strains used in this study

Strain ID[a]	Species	Source	Yr	Resistance phenotype[b]	Resistance genes[c]	Mutation	Purpose
N29710[d]	*C. coli*	Chicken breast	2011	GEN TET	*aadE aad9* ***aph(2″)-Ig*** *aph(3′)-IIIa* bla_{OXA-61} *sat4 tetO*	None	Cloning donor
N20344[d]	*C. coli*	Chicken breast	2009	GEN TET	***aph(2″)-Ic*** *aph(3′)-IIIa* bla_{OXA-61} *tetO*	None	Cloning donor
41912[d]	*C. jejuni*	Human	2008	CIP GEN NAL TET	*aadE aac(6′)-Ie–***aph(2″)-If2** bla_{OXA-61} *tetO*	GyrA86I	Cloning donor
41921[d]	*C. jejuni*	Human	2008	CIP GEN NAL TET	*aadE aad9 aac(6′)-Im* ***aph(2″)-Ib*** *tetO*	GyrA86I	Cloning donor
41945[d]	*C. coli*	Human	2010	CIP AZI CLI ERY TEL GEN NAL TET	*aadE aad9 aac(6′)-Ie–***aph(2″)-Ia aph(2″)-If1** *aph(3′)-IIIa* bla_{OXA-61} *catA tetO*	GyrA86I 23S rRNA 2075G	Cloning donor
41898[e]	*C. coli*	Human	2003	CIP GEN NAL TET	*aadE* ***aac(6′)-Ie–aph(2″)-Ia*** bla_{OXA-61} *tetO*	GyrA86I	Donor
41902[e]	*C. jejuni*	Human	2005	CIP GEN NAL TET	***aph(2″)-If*** *tetO* bla_{OXA-61}	GyrA86I	Cloning donor
41904[e]	*C. coli*	Human	2006	CIP AZI CLI ERY TEL GEN NAL TET	*aadE aad9* ***aph(2″)-If3*** *lnuC aph(3′)-IIIa sat4 tetO*	GyrA86I 23S rRNA 2075G	Cloning donor
41905[e]	*C. jejuni*	Human	2007	CIP AZI CLI ERY TEL GEN NAL TET	*aadE aad9* ***aph(2″)-Ih*** *sat4 aph(3′)-IIIa* bla_{OXA-61} *tetO*	GyrA86I 23S rRNA 2075G	Cloning donor
N18880	*C. jejuni*	Chicken breast	2008	AZT ERY TEL	None	23S rRNA 2075G	Recipient
N46788F	*C. coli*	Cattle	2014	CIP CLI FEN NAL TET	bla_{OXA-61} *aph(3′)-IIIa tetO*	GyrA86I	Recipient

[a]ID, identifier.
[b]GEN, gentamicin; TET, tetracycline; CIP, ciprofloxacin; NAL, nalidixic acid; AZI, azithromycin; CLI, clindamycin; ERY, erythromycin; TEL, telithromycin; FFN, florfenicol.
[c]The genes in bold are aminoglycoside 2″-phosphotransferase [*aph(2″)*] genes.
[d]The *aph(2″)* genes from these isolates were successfully transferred to Gen[s] recipient strains.
[e]The *aph(2″)* genes from these isolates were not successfully transferred to Gen[s] recipient strains.

kanamycin, streptomycin, neomycin, tobramycin, and amikacin, were determined. Agar dilution plates were prepared with concentrations ranging from 0.125 μg/ml to 1,024 μg/ml. The MIC was recorded as the lowest concentration of antimicrobial agent that completely inhibited the growth of an organism after incubation at 35°C for 16 to 20 h, according to guidelines of the Clinical and Laboratory Standards Institute (CLSI) (6)

For conjugation, nine Gen[r] *Campylobacter* strains that carried variants of *aph(2″)* genes were used as donor strains, including four *C. jejuni* (41912, 41921, 41902, and 41905) and five *C. coli* (41945, 41898, 41904, N29710, and N20344) strains. Two gentamicin-susceptible (Gen[s]) strains, *C. jejuni* N18880 and *C. coli* N46788F, were used as recipient strains (Table 1). Conjugation was performed as described by Chen et al. (7). Successful transconjugants were then confirmed by antimicrobial susceptibility testing (AST) and whole-genome sequence (WGS) analysis (4, 8).

AST showed that the expression of the nine *aph* genes in *E. coli* resulted in various levels of resistance to gentamicin, kanamycin, and tobramycin. The MICs from different clones ranged from 16 to 64 μg/ml for gentamicin, 64 to 512 μg/ml for kanamycin, and 8 to 128 μg/ml for tobramycin. Comparing the MICs of *E. coli* that carry pBluescript:: *aph(2″)* to the control strain, there were 32- to 128-, 64- to 512-, and 16- to 256-fold increases in the MICs to gentamicin, kanamycin, and tobramycin, respectively (Table 2). The MIC differences from nine clones to the same drug may explain the diversity of this family. Furthermore, three clones carrying *aph(2″)-Ia*, *aph2″-If3*, and *aph2″-Ig* showed 8-

TABLE 2 MICs of six aminoglycosides for *E. coli* DH5α expressing the nine *aph(2″)* genes

Bacterial strain name	*aph* genes in pBluescript	MIC (μg/ml)					
		Gentamicin	Kanamycin	Tobramycin	Amikacin	Neomycin	Streptomycin
DH5a	pBluescript	0.5	1	0.5	1	1	2
E. coli-Ia	*aac(6′)-Ie–aph(2″)-Ia*[a]	64	512	128	8	0.5	2
E. coli-Ib	*aph(2″)Ib*	16	64	16	1	1	2
E. coli-Ic	*aph(2″)-Ic*	32	64	16	0.5	0.5	2
E. coli-If	*aph(2″)If*	32	128	16	2	1	4
E. coli-If1	*aph(2″)-If1*	32	128	16	2	1	2
E. coli-If2	*aac(6′)-Ie–aph(2″)-If2*[a]	32	128	32	2	1	2
E. coli-If3	*aph(2″)-If3*	32	128	8	8	0.5	2
E. coli-Ig	*aph(2″)-Ig*	32	128	16	16	1	2
E. coli-Ih	*aph(2″)-Ih*	64	128	16	1	0.5	2

[a]For bifunctional aminoglycoside resistance genes, only aminoglycoside phosphotransferase genes [*aph(2″)-Ia* and *aph(2″)-If2*] were cloned to the pBluescript vector.

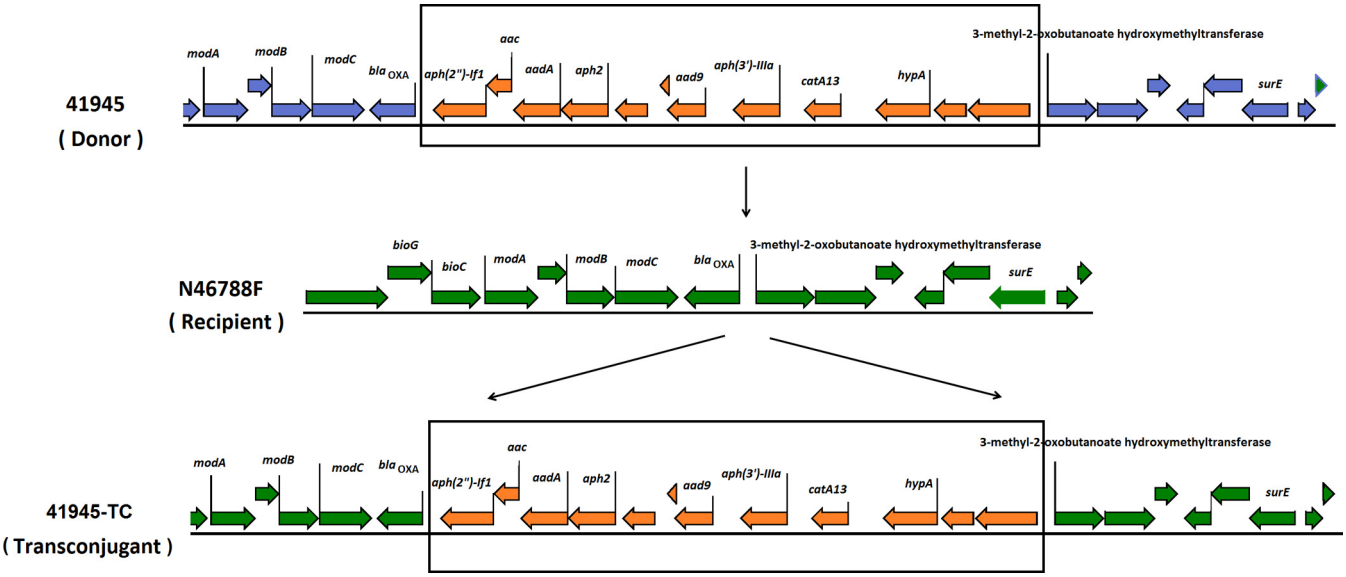

FIG 1 The chromosome-carried Gen^r island integrated into the chromosome of Gen^s isolate through conjugation.

to 16-fold increases in their MICs to amikacin compared to those of the *E. coli* control strain. None of the nine *aph* gene clones showed resistance to streptomycin or neomycin (Table 2).

A similar study conducted by Toth et al. showed that when *aph(2″)-Ia* and *aph(2″)-If* were cloned in pET22b(+) vector and expressed in *E. coli* BL21, the MICs increased 32- to 128-fold to kanamycin, tobramycin, and gentamicin. There was only a 1- to 2-fold increase in the MICs to neomycin and amikacin compared with control strain *E. coli* JM83 (2). Our results agreed with their findings for these two antimicrobial agents, except that pET22b::*aph(2″)-Ia* expressed in *E. coli* JM83 had a 4-fold lower MIC to tobramycin (32 μg/ml) than to pBluescript::*aph(2″)-Ia* expressed in *E. coli* DH5α (128 μg/ml). The difference in MICs to tobramycin between these two experiments could be due to the use of different cloning vectors and expression strains of *E. coli*, as well as the use of different AST methods.

The conjugation study showed that *aph(2″)-Ib* (41921), *aph(2″)-Ic* (N20344), *aph(2″)-If1* (41945), *aph(2″)-If2* (41912), and *aph(2″)-Ig* (N29710) were successfully transferred to Gen^s strains, either in *C. jejuni* N18880 or *C. coli* N46788. Strain 41945 carried two *aph(2″)* genes, bifunctional *aac(6′)-Ie–aph(2″)-Ia* and monofunctional *aph(2″)-If1*, and only *aph(2″)-If1* was transferred based on the comparative genomic analysis. The other four *aph(2″)* genes, including three monofunctional *aph(2″)-If* (41902), *aph(2″)-If3* (41904), *aph(2″)-Ih* (41905) genes and one bifunctional *aac(6′)-Ie–aph(2″)-Ia* (41898) gene, were not transferred to Gen^s strains (Table 1).

WGS analysis of donors, recipients, and transconjugants showed that transferred *aph(2″)* genes, including *aph(2″)-Ib*, *aph(2″)-Ic*, and *aph(2″)-Ig*, and *aac(6′)-Ie–aph(2″)-If2*, are located on plasmids. However, the *aph(2″)-If1* gene from strain 41945, which is located on a chromosome, also was transferred. Comparative genomic analysis of donor (41945), recipient (N46788F), and transconjugant (41945-TC) strains showed that the aminoglycoside resistance island from the donor chromosome was integrated into the chromosome of recipient cells (41945-TC) through a recombination event (Fig. 1). WGS data showed that *aadE*, *aad9*, *sat4*, and *aphA-3*, one of the *aph(2″)* genes, and the *tetO* genes were often clustered together, forming a resistance island located either on the chromosome or the pTet plasmid (4, 7). Similar aminoglycoside resistance genomic islands were found either on the plasmid pCG8245 of *C. jejuni* or in the chromosome of *C. coli* SX81 (9, 10).

In summary, all nine variants of *aph(2″)* genes are responsible for resistance to

gentamicin, kanamycin, and tobramycin but not to neomycin or streptomycin. Three variants, including *aph(2″)-Ia*, *aph(2″)-If3*, and *aph(2″)-Ig*, showed decreased susceptibility to amikacin. Both the plasmid- and chromosome-carried *aph(2″)* gene can be transferred by conjugation. This study highlights the need for continuous monitoring of emergent resistance genes in foodborne pathogens.

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