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Characterization of Two Plasmids from *Campylobacter jejuni* Isolates That Carry the *aphA*-7 Kanamycin Resistance Determinant

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Two small plasmids of 11.5 and 9.5 kb, each carrying an *aphA*-7 kanamycin phosphotransferase gene, were studied. The MICs of kanamycin for the two human *Campylobacter jejuni* isolates harboring the plasmids were 10,000 and 5,000 μg/ml, while the MICs of amikacin were 32 and 8 μg/ml, respectively. The MICs of gentamicin and tobramycin were ≤2 μg/ml for both isolates. The restriction endonuclease maps of the plasmids were similar, with the larger plasmid showing two discrete regions of additional DNA. When the *aphA*-7 gene from each plasmid was cloned into pBR322, the *aphA*-7 gene expressed the kanamycin resistance phenotype in *Escherichia coli*. For transformants containing the cloned *aphA*-7 gene, kanamycin MICs were ≥128 μg/ml. The *aphA*-7 gene was also subcloned from the plasmid pFKT4420 into the *E. coli-Streptococcus* shuttle vector pDL278 and was transformed into *Streptococcus gordonii* Challis. For streptococcal transformants containing the novel plasmid, kanamycin MICs were 4,000 μg/ml. In the presence of a tetracycline resistance plasmid, both small plasmids could be mobilized during conjugal matings to *Campylobacter coli* recipients.

Campylobacter species are common pathogens of humans and animals (1, 13, 14). *Campylobacter jejuni*, in particular, is known to be a frequent cause of diarrheal illness in humans (1). Campylobacters can harbor a variety of resistance determinants (19), and plasmid-mediated resistance, particularly that to tetracycline, is common (16, 20, 21, 24). Previously, we reported the presence of R plasmids encoding both tetracycline and kanamycin resistance (Km^r) in isolates of *C. jejuni* and *Campylobacter coli* (22). While all of the tetracycline-resistant (Tc^r) isolates hybridized with a probe specific for the *tetO* gene (6), only 9 of the 11 Km^r isolates tested hybridized with a probe directed against the *aphA*-3 aminoglycoside phosphotransferase gene, the kanamycin resistance determinant most commonly reported for *Campylobacter* isolates (5, 15, 26). Two strains appeared to harbor novel resistance determinants that failed to hybridize with probes for the *aphA*-1, *aphA*-2, and *aphA*-3 genes. One of the two novel determinants, the *aphA*-7 gene, was cloned in *Escherichia coli* and its DNA sequence was determined (23). An open reading frame of 753 bases encoding a protein of 29 kDa was noted. A DNA probe specific for the *aphA*-7 gene identified a second isolate of *C. jejuni* that carried this determinant.

This study was undertaken to (i) characterize the plasmids encoding the *aphA*-7 gene by comparing their restriction endonuclease maps, (ii) determine the ability of the plasmids to be mobilized during conjugation to a *Campylobacter* recipient, and (iii) assess the ability of the *aphA*-7 gene to be expressed in a streptococcal host. Here, we report the results of those studies.

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MATERIALS AND METHODS

Bacterial strains and growth conditions. The strains and constructs used in this study are described in Table 1. *C. jejuni* PS1178 was isolated in Seattle, Wash., in 1982 from a human with diarrhea (2, 22). This organism carries two plasmids of 45 and 11.5 kb (pFKT1178 and pFKT4497, respectively) and is a Lior 16 serotype. The smaller plasmid was previously reported to be 14 kb (22). *C. jejuni* PS4099 was isolated in Athens, Ga., in 1984, also from a human with diarrhea. This isolate harbors a single plasmid of 9.5 kb (pFKT4099) and is a Lior serotype 11. The latter strain was provided by C. Patton (Centers for Disease Control, Atlanta, Ga.). *C. coli* PS1068, a plasmid-free isolate that is streptomycin and erythromycin resistant (Sm^r and Em^r, respectively) but that is susceptible to kanamycin (Km^s) and tetracycline (Tc^s) (24), was used as a conjugal recipient. *C. coli* PS4497 is a transconjugant resulting from the mating of *C. jejuni* PS1178 and *C. coli* PS1068. It contains only the 11.5-kb plasmid encoding Km^r. PS4462 was constructed by mating PS1025, a strain containing a 48-kb plasmid, pFKT1025 (24), with PS4099, and selecting for both Tc^r and Km^r. PS4462 contains the 48-kb *tetO* plasmid pFKT1025 and the 9.5-kb Km^r plasmid pFKT4099. This strain, in turn, was mated with PS1068, with selection for Km^r and Sm^r, to produce PS4467, which contains both plasmids, and PS4466, which contains only the 9.5-kb plasmid.

Columbia blood agar plates (Prepared Media Labs, Tualatin, Oreg.) were used for propagation of all *Campylobacter* strains. Cultures of *C. jejuni* and *C. coli* were incubated at 42°C in a microaerobic environment that was produced by placing a CampyPak-Plus gas generator envelope (Becton Dickinson Microbiology Systems, Cockeysville, Md.) in an anaerobe jar. *E. coli* DH5α was grown at 37°C in L broth (17). *Streptococcus gordonii* Challis DL1 was grown statically at 37°C in brain heart infusion broth (Difco) or on

TABLE 1. Bacterial strains used in this study

Organism and strain	Plasmid(s)	Phenotype[a]	Description or reference
C. jejuni			
PS1178	pFKT1178, pFKT4497	Km^r Tc^r	22
PS4099	pFKT4099	Km^r	This study
PS1025	pFKT1025	Tc^r	24
PS4462	pFKT1025, pFKT4099	Km^r Tc^r	PS4099 × PS1025
C. coli			
PS1068	None	Em^r Sm^r	24
PS4497	pFKT4497	Km^r Sm^r	PS1178 × PS1068
PS4466	pFKT4099	Km^r Sm^r	PS4462 × PS1068
PS4467	pFKT1025, pFKT4099	Km^r Sm^r	PS4462 × PS1068
E. coli			
PS4492	pFKT4492	Km^r	pBR322 + 4.26-kb *Hin*dIII fragment of pFKT4099
PS4363	pFKT4363	Km^r	pBR322 + 9.40-kb *Eco*RV fragment of pFKT4497
PS4420	pFKT4420	Km^r	*Eco*RV-*Aha*III deletion of pFKT4363
S. gordonii			
DL1	pDL278		7
PS5001	pFKT5001	Km^r	pDL278 + *Eco*RV-*Dra*I fragment of pFKT4420

[a] Em^r, erythromycin resistance; Km^r kanamycin resistance; Sm^r, streptomycin resistance; Tc^r, tetracycline resistance.

Trypticase agar (Difco) containing 5% sheep blood in an atmosphere containing 5% CO_2 (8).

Conjugation experiments. Conjugations were performed on filters by the procedure of Kotarski et al. (4). Briefly, 1 ml of the donor strain containing 10^7 CFU suspended in brucella broth and 1 ml of the recipient strain containing approximately 10^8 CFU were centrifuged for 2 min in 1.5-ml microcentrifuge tubes, resuspended in 50 μl of brucella broth (Difco), and placed together on a 0.2-μm-pore-size Nuclepore filter resting on a blood agar plate. After 48 h of incubation under microaerobic conditions, the filter was placed in 1 ml of brucella broth and vortexed. Samples of 0.1 ml were plated onto the selective media described below. Plates were incubated under appropriate conditions and were examined daily for up to 7 days. Antimicrobial concentrations used for selection were 15 μg/ml for tetracycline, 30 μg/ml for kanamycin, and 50 μg/ml for streptomycin. Suspected transconjugants were subcultured onto antimicrobial agent-containing media to confirm their stabilities and were screened for the presence of plasmid DNA as described previously (24).

Susceptibility testing methods. Susceptibility testing was performed by an agar dilution method as described previously (24). Twofold serial dilutions of kanamycin (Sigma), amikacin (Bristol Laboratories, Syracuse, N.Y.), tobramycin (Sigma), and gentamicin (Sigma) were prepared in Mueller-Hinton agar (Difco). *Campylobacter* strains were grown for 24 h on a blood agar plate as described above, suspended in brucella broth to the density of a 1.0 MacFarland standard, and diluted 1:20 in phosphate-buffered saline (pH 7.0). Antimicrobial agent-containing plates were inoculated by using a multipoint replicator as described previously (24). Plates were incubated microaerobically at 37°C for 48 h. *S. gordonii* strains were tested in a similar fashion, using cation-adjusted Mueller-Hinton broth incubated in an atmosphere containing 5% CO_2 for 24 h.

E. coli DH5α and the quality control strains *Staphylococcus aureus* ATCC 25923 and *E. coli* ATCC 25922 were suspended in brucella broth to the density of a 0.5 MacFarland standard and were then diluted and inoculated onto agar as described above. MICs were determined after 24 h of aerobic incubation at 37°C.

Cloning experiments. A 4.26-kb *Hin*dIII fragment from pFKT4099, which contains the *aphA*-7 gene, was mixed with *Hin*dIII-digested pBR322 DNA, and the mixture was incubated overnight at 16°C in the presence of T4 ligase (Bethesda Research Laboratories, Gaithersburg, Md.). The ligation mixture was used to transform *E. coli* DH5α, with selection for Km^r (30 μg/ml), as described previously (22). One of the Km^r transformants obtained was designated PS4492. The chimeric plasmid pFKT4363 and its *Aha*III deletion derivative pFKT4420, each of which contained the *aphA*-7 gene from PS4497, have been described previously (23).

To clone the *aphA*-7 gene into the streptococcal shuttle vector pDL278 (7), pFKT4420 was digested with *Dra*I (*Aha*III) and *Eco*RV, and pDL278 was digested with *Hin*cII. The digests were mixed in a 1:5 molar ratio of pDL278 to pFKT4420, and the mixture was treated with T4 ligase (Bethesda Research Laboratories) according to the directions of the manufacturer. The reaction mixture was used to transform *E. coli* DH5α, with selection for spectinomycin (50 μg/ml) and kanamycin (50 μg/ml) resistance, in agar containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (17). Transformants were screened for Km^r and plasmids with inserts of approximately 4.5 kb. DNA from one such chimeric plasmid was used to transform *S. gordonii* Challis, with selection for Km^r (250 μg/ml) on brain heart infusion agar as described previously (6). One transformant, PS5001, containing the chimeric plasmid pFKT5001 was selected for further study.

RESULTS

Characterization of the plasmids. The restriction endonuclease maps of the 11.5-kb plasmid pFKT4497, which was originally obtained from *C. jejuni* PS1178, and the 9.5-kb plasmid pFKT4099 from PS4099 were constructed and are shown in Fig. 1. The plasmids, although slightly different in size, had similar restriction endonuclease maps. The *Cla*I

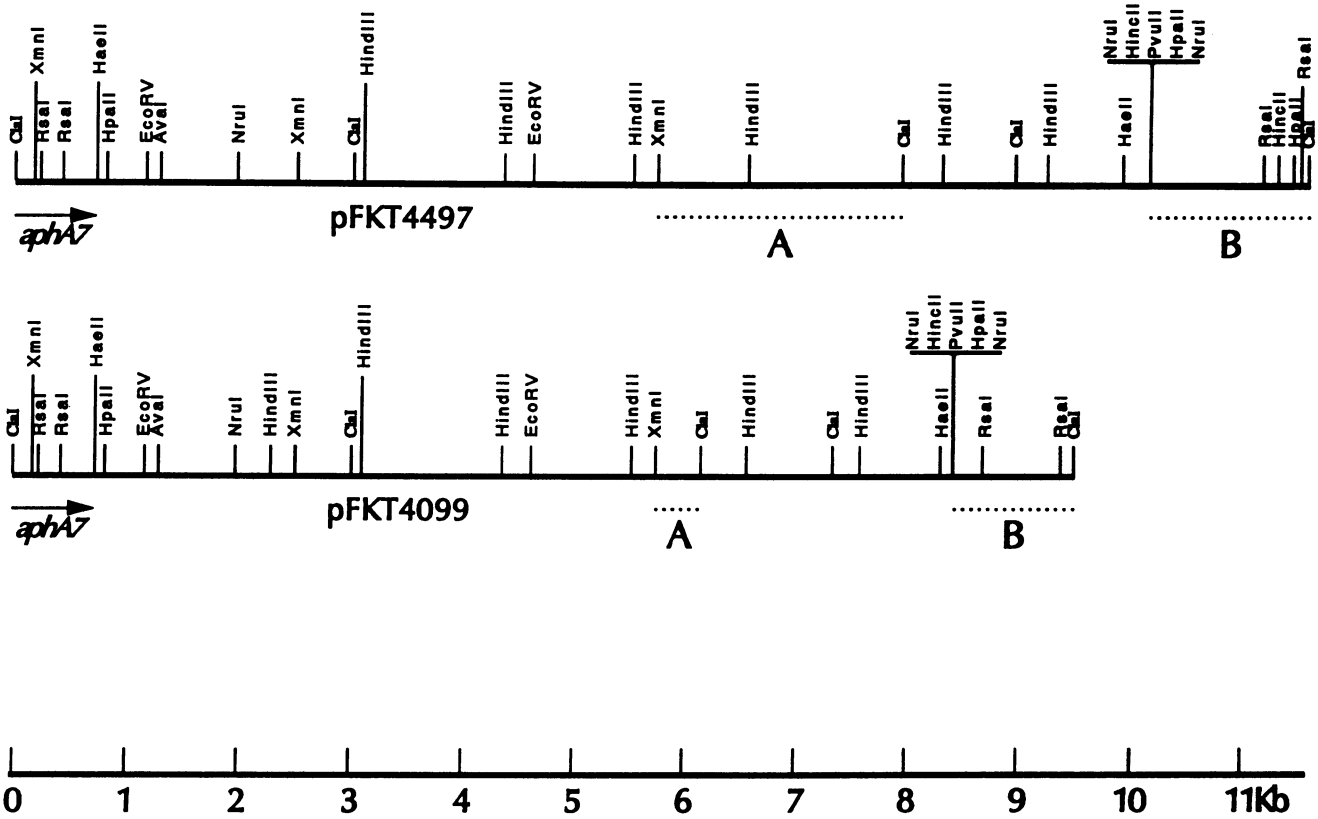

FIG. 1. Restriction endonuclease maps of two *aphA*-7-encoding plasmids recovered from two different isolates of *C. jejuni*. The regions of diversity between the two maps are designated A and B. The exact order of each of the restriction sites on the *Nru*I-*Nru*I fragment could not be determined. The size scale is in kilobases.

site at position 0 on the map of both plasmids is located 12 bp into the open reading frame of the *aphA*-7 gene (23). There are two major regions of divergence between the two plasmids (Fig. 1, regions A and B) and an additional *Hin*dIII site at approximately 2.3 kb on the pFKT4099 map. Because of the tight clustering of restriction sites at the right ends of the plasmids, the exact orientations of the cleavage sites could not be determined.

Subcloning of the *aphA*-7 gene in *E. coli* and *S. gordonii*. A 4.26-kb *Hin*dIII fragment containing the *aphA*-7 gene from pFKT4099 (23) was cloned into the *Hin*dIII site of the vector pBR322 by using *E. coli* DH5α Km^s as the host. One Km^r transformant, designated PS4492, was chosen for further study. The plasmid pFKT4363, which contained the *aphA*-7 gene from plasmid pFKT4497 from *C. jejuni* PS1178, and the plasmid pFKT4420, which was derived by deletion of several *Aha*III fragments from pFKT4363, both of which mediate Km^r, have been described previously (23).

The *aphA*-7 gene from pFKT4420 was cloned into the streptococcal shuttle vector pDL278 in *E. coli*. Plasmid DNA was obtained from an *E. coli* transformant and was used to transform *S. gordonii* Challis. A Km^r transformant, designated PS5001, contained the recombinant plasmid pFKT5001.

Conjugation studies. PS4099 containing the 9.5-kb Km^r plasmid was mated with *C. jejuni* PS1025, which carries the 48-kb tetracycline resistance plasmid pFKT1024 (24). A transconjugant that expressed both the Tc^r and Km^r resistance phenotypes was recovered (PS4462) and was shown to harbor both the 48- and 9.5-kb plasmids (Table 1). This transconjugant served as a conjugal donor for subsequent matings with *C. coli* PS1068. Both plasmids were transferred during overnight matings to *C. coli*. One transconjugant that contained both plasmids was selected and was designated PS4467. A second transconjugant that harbored only the 9.5-kb Km^r resistance plasmid (PS4466) was recovered. The transfer of pFKT4497 from PS1178 to *C. coli* PS1068 has been reported previously (22).

MICs. The MICs of kanamycin, amikacin, tobramycin, and gentamicin for the two original *C. jejuni* isolates PS1178 and PS4099, the *Campylobacter* transconjugant PS4467 containing the *aphA*-7 plasmid, and the *E. coli* and *S. gordonii* transformants containing the *aphA*-7 gene are given in Table 2. For all of the *Campylobacter* strains that harbored an *aphA*-7 gene, the MICs of kanamycin were ≥5,000 μg/ml. Although the MICs of amikacin remained at relatively low levels, they increased 16- to 32-fold for the *Campylobacter* transconjugant and *E. coli* PS4420. This increase in the MIC of amikacin is consistent with the high level of amikacin-modifying activity previously shown for the *aphA*-7 gene (22). For *E. coli* PS4363, the MICs of tobramycin and gentamicin, in addition to those of kanamycin and amikacin, exhibited a modest but consistent increase (Table 2). There was approximately a 10-fold difference in the MIC of kanamycin for the *E. coli* strain containing the *aphA*-7 gene from pFKT4497 (PS4420) and the determinant from pFKT4099 (PS4492). The reasons for this are unclear, but they may be related to differences in the promoters of the two *aphA*-7 genes or to the presence of other promoters on the cloning vector. The 4,000-μg/ml MIC of kanamycin for strain PS5001

TABLE 2. MICs of aminoglycosides for bacterial species

Organism and strain	MIC (μg/ml)			
	Kanamycin	Amikacin	Tobramycin	Gentamicin
C. jejuni				
PS4099	10,000	32	2	0.5
PS1178	5,000	8	2	0.5
C. coli				
PS1068	4	1	1	0.5
PS4467	5,000	16	2	0.5
E. coli				
DH5α	0.5	0.25	0.12	0.06
PS4492 (*aphA*-7)	128	0.5	0.12	0.06
PS4363 (*aphA*-7)	512	2	0.5	0.12
PS4420 (*aphA*-7)	1,024	8	1	0.06
S. gordonii				
DL1(pDL278)	50	25	12	4
PS5001(pFKT5001)	4,000	50	12	4

demonstrates that the *aphA*-7 gene is expressed in this streptococcal host.

DISCUSSION

Kanamycin resistance was first reported for an isolate of *C. coli* by Lambert and colleagues (5) in 1985. The gene responsible for resistance was *aphA*-3, a resistance determinant common in staphylococci and streptococci (3, 5, 10, 12, 25). Subsequently, Ouellette et al. (9) noted the presence of the *aphA*-1 gene in a *Campylobacter*-like isolate. Then, in 1988, we reported the presence of a novel Km^r gene in an isolate of *C. jejuni* from Seattle, Wash. (22). We characterized the two plasmids obtained from unrelated *C. jejuni* isolates that carry the *aphA*-7 kanamycin phosphotransferase determinants. The restriction endonuclease maps of both plasmids were similar, suggesting that they were derived from a common ancestral plasmid by either the insertion or the deletion of genetic material. The prevalence of these small resistance plasmids in campylobacters is unknown; similar plasmids have not been reported by other investigators.

Previously, we speculated that the *aphA*-7 gene, which has a guanine-plus-cytosine ratio similar to that of the *Campylobacter* chromosome (11) and a typical gram-negative ribosomal binding site (23), is indigenous to campylobacters. This is in contrast to the *aphA*-3 Km^r gene and the *tetO* resistance gene, which are believed to have entered the *Campylobacter* gene pool from gram-positive sources (5, 6, 12, 18, 26). While the *aphA*-3 gene frequently is found in campylobacters on large plasmids that also encode a *tetO* determinant (4, 16, 22), the *aphA*-7 determinants we studied were located on small plasmids that apparently do not encode other resistance determinants. Both of the Km^r plasmids could be mobilized to a recipient strain of *C. coli* when a large plasmid carrying the *tetO* gene was present in the same strain. While PS1178 contains such a plasmid, it was necessary to introduce pFKT1025 into PS4099 to prove that the smaller plasmid, pFKT4099, also is capable of being mobilized. This suggests that the insertions (or deletions) of material took place outside the regions necessary for mobilization.

The *aphA*-7 gene, after being cloned into the appropriate vectors, was shown to be expressed in both *E. coli* and *S. gordonii* transformants, demonstrating that the *aphA*-7 gene has a host range that includes both gram-positive and gram-negative organisms. Interestingly, a recent survey of 500 enterococcal and 500 staphylococcal isolates by Ounissi et al. (10) showed that virtually 100% of the Km^r isolates examined by DNA hybridization encoded an *aphA*-3 gene; none of the isolates carried the *aphA*-7 gene. This further supports the hypothesis that the *aphA*-7 gene is indigenous to campylobacters and was not acquired from a gram-positive donor. Although the *aphA*-7 gene could have originated in another gram-negative donor, the low G+C ratio of the gene, which matches that of the *C. jejuni* chromosome, argues against this possibility.

We noted considerable variability of the kanamycin MICs among the strains and constructs containing the *aphA*-7 gene. For the *E. coli* isolate harboring the cloned *aphA*-7 gene from pFKT4497 (PS4363), the MIC was four times greater than that for the isolate carrying the gene from pFKT4099 (PS4492) (128 versus 512 μg/ml), while the MIC of kanamycin for *E. coli* PS4420 was 1 dilution higher (1,028 μg/ml). These results may be due to differences within the promoters of the two *aphA*-7 genes or to the effects on other promoters present on the plasmids. Both pFKT4492 and pFKT4363 contain a functional ampicillin resistance determinant, while pFKT4420 does not. Comparison of the nucleotide sequences of the two *aphA*-7 promoters will be required to address this question more fully. The differences between the kanamycin MICs for the *C. jejuni* and the *E. coli* strains carrying the cloned *aphA*-7 genes suggest that the promoters are read more efficiently in *C. jejuni*. The ultimate explanation for these differences, however, awaits further study.

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