ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Nov. 2005, p. 4681–4688 Vol. 49, No. 11
0066-4804/05/$08.00+0 doi:10.1128/AAC.49.11.4681–4688.2005

DNA Sequence and Comparative Genomics of pAPEC-O2-R, an Avian Pathogenic *Escherichia coli* Transmissible R Plasmid

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Received 9 May 2005/Returned for modification 13 July 2005/Accepted 9 August 2005

In this study, a 101-kb IncF plasmid from an avian pathogenic *Escherichia coli* (APEC) strain (APEC O2) was sequenced and analyzed, providing the first completed APEC plasmid sequence. This plasmid, pAPEC-O2-R, has functional transfer and antimicrobial resistance-encoding regions. The resistance-encoding region encodes resistance to eight groups of antimicrobial agents, including silver and other heavy metals, quaternary ammonium compounds, tetracycline, sulfonamides, aminoglycosides, trimethoprim, and beta-lactam antimicrobial agents. This region of the plasmid is unique among previously described IncF plasmids in that it possesses a class 1 integron that harbors three gene cassettes and a heavy metal resistance operon. This region spans 33 kb and is flanked by the RepFII plasmid replicon and an assortment of plasmid maintenance genes. pAPEC-O2-R also contains a 32-kb transfer region that is nearly identical to that found in the *E. coli* F plasmid, rendering it transferable by conjugation to plasmid-less strains of bacteria, including an APEC strain, a fecal *E. coli* strain from an apparently healthy bird, a *Salmonella enterica* serovar Typhimurium strain, and a uropathogenic *E. coli* strain from humans. Differences in the G+C contents of individual open reading frames suggest that various regions of pAPEC-O2-R had dissimilar origins. The presence of pAPEC-O2-R-like plasmids that encode resistance to multiple antimicrobial agents and that are readily transmissible from APEC to other bacteria suggests the possibility that such plasmids may serve as a reservoir of resistance genes for other bacteria of animal and human health significance.

Antimicrobial resistance among bacterial pathogens of food animals can complicate veterinary therapy. Resistant animal pathogens may also be a threat to human health if these resistant bacteria enter the food supply or otherwise serve as reservoirs of resistance genes for human pathogens. Transmissible R plasmids that encode multidrug resistance would seem a likely means by which animal pathogens could acquire resistance genes or transmit them to human pathogens. This study examines an R plasmid encoding multidrug resistance in an avian pathogenic *Escherichia coli* (APEC) isolate. APEC strains are important and prevalent bacterial pathogens of poultry (3) and are frequently found to be resistant to multiple antimicrobial agents (21, 37), including ampicillin, tetracycline, aminoglycosides, fluoroquinolones, quaternary ammonium compounds, and heavy metals (37). Genes encoding such resistance are often found on large, transmissible R plasmids (20). Not surprisingly, multidrug-resistant APEC strains often carry conjugative plasmids (8). Interestingly, plasmids have been shown to be transferable from poultry to human isolates (23), suggesting that APEC strains and their plasmids might serve as reservoirs of resistance genes for bacteria that affect public health. In the present study, the first complete sequence of a transmissible APEC R plasmid is presented and analyzed. Additionally, an effort was made to determine the transmissibility of this plasmid to other bacteria found in poultry and to an *E. coli* strain from human disease in order to assess the potential of this plasmid to serve as a reservoir of resistance genes for pathogens of animal and human health significance.

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MATERIALS AND METHODS

Bacterial strains and plasmids. The original source of pAPEC-O2-R, the plasmid sequenced in this study, was a wild-type avian *E. coli* isolate named APEC O2, with the "O2" in its name referring to its serogroup. APEC O2 was isolated from a chicken clinically diagnosed with colibacillosis. All strains were grown at 37°C in Luria-Bertani broth medium (LB broth; Difco Laboratories, Detroit, MI), supplemented as needed with antimicrobial agents at the following concentrations: ampicillin, 100 μg/ml; tetracycline, 12.5 μg/ml; and/or nalidixic acid, 30 μg/ml. All bacterial strains were stored at −70°C in brain heart infusion broth (Difco Laboratories) with 10% glycerol until they were used (32). The recipients used in the conjugation studies included avian pathogenic *E. coli* strain 419; an avian fecal commensal *E. coli* (AFEC) isolate from an apparently healthy chicken, A3; a uropathogenic *E. coli* (UPEC) strain, 2000-1; and *Salmonella enteric* serovar Typhimurium strain 475. Additional details about these recipients are provided in Table 1.

Antimicrobial susceptibility testing. The donor strain possessing pAPEC-O2-R, the recipient strains, and their transconjugants were examined for resistance to ampicillin, tetracycline, chloramphenicol, streptomycin, spectinomycin, sulfisoxazole, gentamicin, trimethoprim, silver nitrate, and benzalkonium chloride by disk diffusion assays. These assays were performed with BBL Sensi-Disk antimicrobial susceptibility test disks (BD, Franklin Lakes, NJ), in accordance with the CLSI (formerly the NCCLS) standard Kirby-Bauer disk diffusion method (28, 29). Briefly, Mueller-Hinton agar plates (Difco Laboratories) were swabbed with *E. coli* cultures grown to a McFarland standard of 0.5. Zones of inhibition were measured in millimeters (including disk diameter) and were categorized as sensitive or resistant according to the CLSI breakpoints.

Disk diffusion was also used to test the *E. coli* isolates for their susceptibilities to benzalkonium chloride and silver nitrate. For these compounds, sterile 5.5-cm filter paper disks (Fisher Scientific) were placed on Mueller-Hinton agar plates swabbed with *E. coli* cultures grown to a McFarland standard of 0.5. Ten microliters of either of these compounds was then pipetted onto an individual disk from the following stock concentrations: 0.1 M silver nitrate and 0.1 M benzalkonium chloride. All plates were incubated overnight at 37°C, and zones of inhibition were measured in millimeters and compared to known positive and

TABLE 1. Bacterial strains used in matings with APEC O2

Name	Source	Mating frequency with APEC O2[a]	Drugs to which resistance was acquired by transconjugant[b]
APEC 419	Lesion of chicken with colibacillosis	2.3×10^{-2}	Ap Te St Su Gn Tm An Bc
AFEC A3	Feces of healthy chicken	1.7×10^{-2}	Ap Te St Su Gn Tm An Bc
UPEC 2000–1	Human urinary tract infection	2.1×10^{-2}	Ap Te St Su Gn Tm An Bc
E. coli DH5α	NA[c]	1.9×10^{-2}	Ap Te St Su Gn Tm An Bc
S. enterica serovar Typhimurium 475	Centers for Disease Control and Prevention	2.5×10^{-2}	Ap Te St Su Gn Tm An Bc

[a] Mating frequencies are expressed as the proportion of transconjugants to recipients.
[b] Ap, ampicillin; Te, tetracycline; St, streptomycin; Su, sulfisoxazole; Gn, gentamicin; Tm, trimethoprim; An, silver nitrate; Bc, benzalkonium chloride.
[c] NA, not available.

negative controls on the following day. The positive control used to measure susceptibility to benzalkonium chloride and silver nitrate was APEC O2, which is resistant to these agents. *E. coli* DH5α, which is sensitive to these two antimicrobial agents, was used as a negative control (31). Strains were classified as sensitive or resistant to benzalkonium chloride and silver nitrate based on comparison to those of known positive and negative controls.

Bacterial conjugations and DNA isolation. The transmissibility of pAPEC-O2-R was determined by mating APEC O2 with several plasmid-less bacteria (Table 1) by using a previously described protocol (19). Mating mixtures were incubated overnight at 25°C, 37°C, and 42°C; and transconjugants were selected on Mueller-Hinton agar (Difco Laboratories) containing appropriate antibiotics. Putative transconjugants were verified by their antimicrobial resistance profiles, plasmid contents, and gene contents, as determined by the use of a series of multiplex PCR protocols described previously (30). Mating frequencies were determined by measuring the proportion of transconjugant colonies to recipient colonies. The plasmid DNA used in this study was obtained from overnight cultures in LB broth containing ampicillin (100 μg/ml), according to the methods of Wang and Rossman (36). Plasmid DNA was separated by horizontal agarose gel electrophoresis (0.7% TAE [Tris-acetate-EDTA]; 3.5 V/cm).

Shotgun library construction and sequencing. Plasmid DNA was sheared, concentrated, and desalted by using standard protocols (31). DNA was end repaired (30 min; 15°C; 100-μl reaction mixture consisting of 2 μg sheared DNA, 15 U T4 DNA polymerase, 10 U *E. coli* DNA polymerase [MBI Fermentas, Vilnius, Lithuania], 500 μM each deoxynucleoside triphosphate, 10 μl Yellow Tango buffer [MBI Fermentas]), desalted, and tailed with an extra A residue (30 min; 50°C; 100-μl reaction mixture consisting of 2 μg sheared DNA; 50 μM each dCTP, dGTP, and dTTP; 2 mM dATP; 20 U *Taq* polymerase [MBI Fermentas], 10 μl Yellow Tango buffer). The A-tailed DNA was then size fractionated by electrophoresis, and the 1.5- to 2.5-kb fraction was isolated and purified by standard methods (31) prior to cloning into pGEM-T (Promega, Madison, WI).

Sequencing was performed by MWG Biotech, Inc. (Hedersberg, Germany). Briefly, plasmid clones were grown for 20 h in 1.8 ml LB broth supplemented with 200 μg/ml ampicillin in deep-well boxes. Plasmid DNA were prepared on a RoboPrep2500 DNA-Prep-Robot (MWG-Biotech, Ebersberg, Germany) by using a NucleoSpin Robot-96 Plasmid kit (Macherey & Nagel, Dueren, Germany) and sequenced from both ends with standard primers by using the BigDye Terminator chemistry (Applied Biosystems, Foster City, CA). The data were collected with ABI 3700 and ABI 3730xl capillary sequencers (Applied Biosystems) and assembled by using the Gap 4 program (5).

Analysis and annotation. Open reading frames (ORFs) in the plasmid sequence were identified by using GeneQuest from DNASTAR (Madison, WI) and GLIMMER 2.02 (11), followed by manual inspection. Translated ORFs were then compared to known protein sequences by using the BLAST program (March 2005 version; National Center for Biotechnology Information). Those with greater than 60% identity were considered matches. Hypothetical proteins with greater than 60% identity to one or more previously published proteins were classified as conserved hypothetical proteins, and ORFs with less than 60% identity to any published sequences were classified as hypothetical proteins. The G+C contents of individual ORFs were analyzed by using GeneQuest (DNASTAR). Insertion sequences and repetitive elements were identified by using IS FINDER (http://www-is.biotoul.fr/). Genomic comparisons of pAPEC-O2-R to similar plasmids were done by using MAUVE alignments (10). Amino acid sequence alignments were performed by using MegAlign (DNASTAR).

Nucleotide sequence accession number. The complete sequence of pAPEC-O2-R was deposited in GenBank under accession number AY214164.

RESULTS

Antimicrobial susceptibility testing. The transconjugant containing pAPEC-O2-R and plasmid donor APEC O2 were resistant to ampicillin, sulfisoxazole, tetracycline, streptomycin, gentamicin, trimethoprim, silver nitrate, and benzalkonium chloride; the recipient, *E. coli* DH5α, was susceptible to all antimicrobial agents tested. APEC O2 was mated to several plasmid-less strains of enteric bacteria, including AFEC A3, APEC 419, *S. enterica* serovar Typhimurium 475, and UPEC 2000-1. All pairings produced transconjugants at similar mating frequencies (Table 1). In each case, the recipients acquired the resistance profiles of the donor (Table 1) and a large plasmid consistent with the size of pAPEC-O2-R.

Sequencing and analysis of pAPEC-O2-R. Three thousand ninety-five shotgun clones of pAPEC-O2-R were arrayed, sequenced, and assembled by using the Gap4 program (5). The assembly resulted in the generation of a complete circular sequence (Fig. 1) of 101,375 bp with approximately 20-fold coverage. pAPEC-O2-R contains 123 predicted ORFs; all coding regions and their closest database matches are provided in Table 2. One hundred eleven of these ORFs showed 60% or greater identity to a previously published sequence. Of these, 82 have a known function, and 29 are conserved hypothetical proteins. The remaining 12 ORFs are classified as hypothetical proteins for which no significant matches in the database were identified. Overall, these ORFs were arranged in distinct regions and encoded antimicrobial resistance, transmissibility, replication, and maintenance (Fig. 1).

Analysis of the coding regions of pAPEC-O2-R revealed a 33,950-bp region containing 15 genes responsible for resistance to at least eight antimicrobial agents (Table 2). This region begins following the *hnh* gene with the start of the *sil* gene cluster, a seven-component system that encodes resistance to silver and other heavy metals (16). Following this cluster is an insertion sequence, IS*26*, that marks the beginning of the *tetAR* complex encoding tetracycline resistance. Immediately following the *tetAR* genes is a 12,282-bp region of pAPEC-O2-R that contains a class 1 integron also found in transposon Tn*21* (24). The class 1 integron of pAPEC-O2-R contains three gene cassettes, including the *catB3*, *aadA5*, and *folA* genes. Following the class 1 integron is Tn*3*, a transposon containing $bla_{\text{TEM-1}}$, a gene encoding a beta-lactamase.

pAPEC-O2-R also contains genes involved in its own maintenance and replication. Near the transfer region are several genes involved in plasmid maintenance, including *hok* and *sok*,

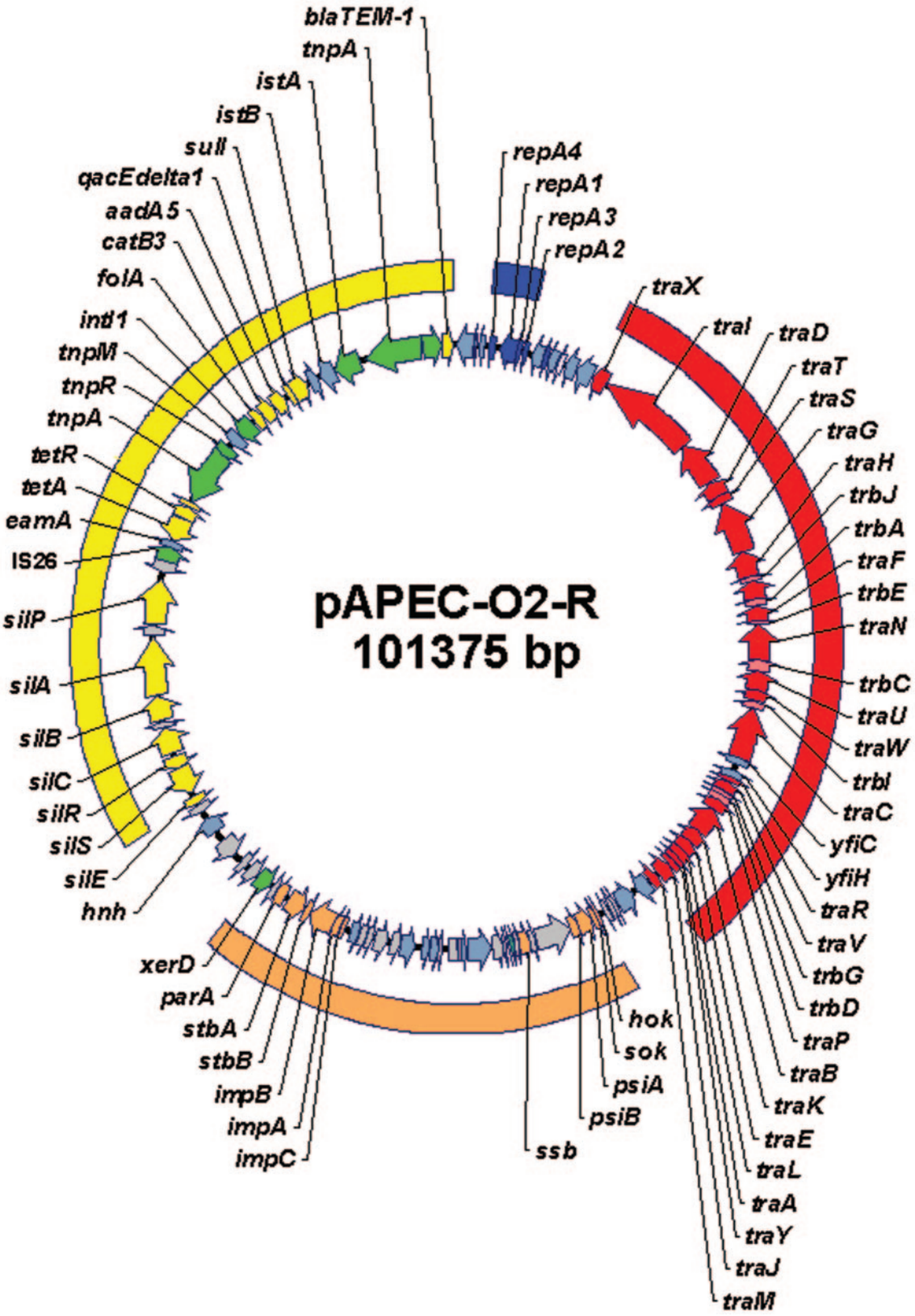

FIG. 1. Circular genetic map of pAPEC-O2-R. Coding regions are indicated by arrows pointing in the direction of transcription. Yellow arrows indicate coding regions involved in antimicrobial resistance, blue arrows indicate coding regions involved in replication, red and pink arrows indicate coding regions involved in plasmid transfer, brown arrows indicate coding regions involved in plasmid maintenance, green arrows indicate mobile elements, blue-gray arrows indicate conserved hypothetical proteins, and gray arrows indicate unknown hypothetical proteins.

TABLE 2. Coding regions of pAPEC-O2-R

Coding sequence	Coordinates	Function of closest protein match	Source	% Identity	GenBank accession no.
yacC	865–17	Exonuclease	*Escherichia coli* plasmid ColIb-P9	98	BAA75091
yacB	1192–911	Unknown	*Escherichia coli* plasmid ColIb-P9	98	BAA75090
yacA	1458–1189	Unknown	*Escherichia coli* plasmid ColIb-P9	97	BAA75089
repA4	2095–1646	Stable inheritance	*Escherichia coli* plasmid R100	100	NP_052991
repA1	3271–2340	Plasmid replication	*Escherichia coli* plasmid B171	98	NP_053107
repA3	3448–3221	Plasmid replication	*Escherichia coli* plasmid TUC100	79	AAM14716
repA2	3788–3531	Negative regulator of plasmid replication	*Escherichia coli* plasmid R100	63	NP_052988
yihA	4618–4028	Unknown	*Escherichia coli* plasmid C15-1a	99	AAR25121
hha	4865–4656	Modulating protein	*Escherichia coli* plasmid R100	100	YP_053130
yigB	5465–4911	Unknown	*Escherichia coli* plasmid C15-1a	100	AAR25120
orf11	5813–5571	Conserved hypothetical protein	*Escherichia coli* plasmid 1658/97	57	AAO49551
finO	6563–5958	Fertility inhibition protein	*Escherichia coli* plasmid R100	100	BAA78888
yieA	7481–6621	Unknown	*Escherichia coli* plasmid C15-1a	100	AAR25115
traX	8292–7540	F pilus acetylation	*Escherichia coli* plasmid R100	97	BAA78886
traI	13582–8306	DNA helicase	*Escherichia coli* plasmid R100	97	NP_052981
traD	15726–13576	Coupling	*Escherichia coli* plasmid R100	97	NP_052980
traT	16803–16027	Surface exclusion and serum resistance	*Escherichia coli* plasmid F	99	BAA97971
traS	17320–16790	Entry exclusion	*Escherichia coli* plasmid F	79	BAA78881
traG	20130–17308	Pilus assembly	*Escherichia coli* plasmid R100	93	NP_052976
traH	21503–20127	Pilus assembly	*Escherichia coli* plasmid F	99	BAA97968
trbJ	21802–21500	Plasmid transfer	*Escherichia coli* plasmid R100	86	NP_052973
trbB	22337–21792	Unknown	*Escherichia coli* plasmid F	97	BAA97965
traQ	22608–22324	Pilus biosynthesis	*Escherichia coli* plasmid F	98	BAA97964
trbA	23074–22727	Unknown	*Escherichia coli* plasmid F	89	BAA97962
traF	23833–23090	Unknown	*Escherichia coli* plasmid F	99	BAA97961
trbE	24083–23826	Unknown	*Escherichia coli* plasmid F	94	BAA97960
traN	25918–24110	Mating pair stabilization	*Escherichia coli* plasmid F	99	BAA97959
trbC	26517–25915	Pilus assembly	*Escherichia coli* plasmid F	99	BAA97958
traU	27554–26562	Pilus assembly	*Escherichia coli* plasmid R100	99	NP_052963
traW	28138–27551	Pilus assembly	*Escherichia coli* plasmid F	99	BAA97956
trbI	28566–28180	Plasmid transfer	*Escherichia coli* plasmid R100	99	NP_052961
traC	31193–28563	Pilus assembly	*Escherichia coli* plasmid F	99	BAA97956
yfiC	31681–31319	Unknown	*Escherichia coli* plasmid R100	79	NP_052959
orf34	31924–31709	Conserved hypothetical protein	*Escherichia coli* plasmid R100	96	NP_052958
yfiA	32477–32004	Unknown	*Escherichia coli* plasmid R100	94	NP_052957
traV	32761–32276	Plasmid transfer	*Escherichia coli* plasmid 1658/97	100	AAO49525
trbG	33524–33273	Plasmid transfer	*Escherichia coli* plasmid F	98	BAA97951
trbD	33828–33521	Plasmid transfer	*Escherichia coli* plasmid F	89	NP_061459
traP	34407–33835	Pilus expression	*Escherichia coli* plasmid ColB2	97	AAB07776
traB	35824–34397	Pilus assembly	*Escherichia coli* plasmid F	100	BAA97948
traK	36552–35824	Pilus assembly	*Escherichia coli* plasmid F	100	BAA97947
traE	37105–36539	Pilus assembly	*Escherichia coli* plasmid F	99	BAA97946
traL	37438–37127	Pilus assembly	*Escherichia coli* plasmid F	100	BAA97945
traA	37812–37453	Plasmid transfer	*Escherichia coli* plasmid 1658/97	97	AAO49517
traY	38166–37846	Plasmid transfer	*Escherichia coli* plasmid ColB4	97	AAB04665
traJ	38853–38167	Plasmid transfer regulation	*Escherichia coli* plasmid R1	98	P05837
traM	39427–39044	Plasmid transfer	*Escherichia coli* plasmid ColB4-K98	98	P18807
ygfA	40351–39758	Unknown	*Escherichia coli* plasmid F	97	BAA97940
ygeB	41469–40648	Unknown	*Escherichia coli* plasmid F	99	BAA97939
orf50	41650–41922	Hypothetical protein			
orf51	42108–41902	Hypothetical protein			
orf52	42356–42144	Hypothetical protein			
orf53	42279–42512	Hypothetical protein			
hok	42956–42798	Postsegregation killing	*Escherichia coli* plasmid R100	100	NP_052939
sok	42988–43224	Postsegregation killing	*Escherichia coli* plasmid R1	100	P13971
psiA	43955–43236	SOS inhibition	*Escherichia coli* plasmid F	100	NP_061443
psiB	44437–43952	SOS inhibition	*Escherichia coli* plasmid F	99	SO1898
orf58	46399–44441	Conserved hypothetical protein	*Escherichia coli* plasmid F	93	BAA75128
ykfF	46748–46464	Unknown	*Escherichia coli* plasmid F	97	AAD47188
ssb	47298–46759	Single-stranded DNA binding	*Escherichia coli* plasmid F	98	BAA97930
orf61	47530–47324	Hypothetical protein			
orf62	47984–47532	Hypothetical protein			
orf63	48148–47985	Hypothetical protein			
ydcA	48712–48149	Unknown	*Escherichia coli* plasmid R100	97	NP_052920
ydbA	50121–48760	Unknown	*Escherichia coli* plasmid R100	99	NP_052919
ydaB	50403–50173	Unknown	*Escherichia coli* plasmid R100	100	NP_052918
orf67	50667–51089	Conserved hypothetical protein	*Escherichia coli* plasmid 1658/97	82	AAO49640

Continued on following page

TABLE 2—*Continued*

Coding sequence	Coordinates	Function of closest protein match	Source	% Identity	GenBank accession no.
orf68	51632–51441	Hypothetical protein			
yfhA	52051–51629	Unknown	*Escherichia coli* plasmid F	96	BAA97928
yciB	52536–52098	Antirestriction protein	*Escherichia coli* plasmid C15-1a	99	NP_957575
orf71	52654–52824	Hypothetical protein			
ychA	53711–52935	Unknown	*Escherichia coli* plasmid R100	97	NP_052912
orf73	54206–53757	Unknown	*Escherichia coli* plasmid O157	93	AAC70143
orf74	54426–54205	Hypothetical protein			
yfeA	55110–54427	DNA methylase	*Escherichia coli* plasmid F	94	BAA97922
orf76	55530–55186	Conserved hypothetical protein	*Shigella flexneri* plasmid WR100	97	CAC05844
yfdA	55931–55494	D-Serine permease	*Escherichia coli* plasmid F	94	BAA97920
yfcB	56419–55913	Glutamine methyltransferase	*Escherichia coli* plasmid F	94	BAA97919
impC	56813–57061	UV protection	*Salmonella enterica* plasmid SC137	100	AAS76415
impA	57058–57495	UV protection	*Salmonella enterica* plasmid SC137	100	AAS76416
impB	57495–58766	UV protection	*Shigella flexneri* SA100 virulence plasmid	100	AAD03593
stbB	59163–58771	Stable plasmid inheritance	*Escherichia coli* plasmid B171	95	NP_053129
stbA	60139–59168	Stable plasmid inheritance	*Escherichia coli* plasmid B171	100	NP_053130
parA	60368–61012	Plasmid partitioning	*Escherichia coli* plasmid B171	99	BAA84904
orf85	61006–61281	Conserved hypothetical protein	*Escherichia coli* plasmid B171	100	NP_053132
rsvB	62201–61419	Resolvase	*Escherichia coli* plasmid B171	88	NP_053133
orf87	62877–62269	Hypothetical protein			
orf88	63439–63035	Hypothetical protein			
orf89	64850–63876	Conserved hypothetical protein	*Klebsiella pneumoniae* plasmid LVPK	97	NP_943494
hnh	65488–66396	Endonuclease	*Klebsiella pneumoniae* plasmid LVPK	99	NP_943492
orf91	67185–66784	Conserved hypothetical protein	*Klebsiella pneumoniae* plasmid LVPK	99	NP_943490
silE	67772–67278	Silver and heavy metal resistance	*Klebsiella pneumoniae* plasmid LVPK	98	NP_943489
silS	69435–67960	Silver and heavy metal resistance	*Klebsiella pneumoniae* plasmid LVPK	100	NP_943488
silR	70108–69428	Silver and heavy metal resistance	*Klebsiella pneumoniae* plasmid LVPK	98	NP_943487
silC	70298–71683	Silver and heavy metal resistance	*Klebsiella pneumoniae* plasmid LVPK	97	NP_943486
orf96	71711–72064	Silver and heavy metal resistance	*Klebsiella pneumoniae* plasmid LVPK	100	NP_941215
silB	72178–73470	Silver and heavy metal resistance	*Klebsiella pneumoniae* plasmid LVPK	98	NP_943483
silA	73481–76627	Silver and heavy metal resistance	*Klebsiella pneumoniae* plasmid LVPK	98	NP_943482
orf99	76714–77154	Conserved hypothetical protein	*Klebsiella pneumoniae* plasmid LVPK	98	NP_943481
silP	77268–79729	Silver and heavy metal resistance	*Klebsiella pneumoniae* plasmid LVPK	98	NP_943480
orf101	80711–80001	Conserved hypothetical protein	*Klebsiella pneumoniae* plasmid LVPK	99	NP_943478
tnpA	80762–81466	IS*26* transposase	*Escherichia coli*	100	CAD43299
pecM	81472–81867	Unknown	*Escherichia coli* plasmid C15-1a	94	NP_957550
tetA	83098–81899	Tetracycline resistance	*Escherichia coli*	99	AAT37966
tetR	83177–82858	Tetracycline repressor	*Escherichia coli*	100	AAT37964
orf106	84128–83886	Relaxase and helicase	*Salmonella enterica* plasmid SC138	98	AAS76290
tnpA	87152–84156	IS*1721* transposase	*Escherichia coli*	99	JQ1477
tnpR	87716–87156	IS*1721* resolvase	*Escherichia coli*	99	CAA46340
tnpM	88476–87892	Tn*21* modulator	*Escherichia coli*	100	AAC33910
intI1	89458–88445	Integrase	*Escherichia coli* plasmid R100	100	NP_052898
folA	89604–90088	Trimethoprim resistance	*Escherichia coli* plasmid R721	99	NP_065309
catB3	90206–90838	Chloramphenicol resistance	*Escherichia coli* plasmid HSH2	100	AAP20921
aadA5	90896–91684	Streptomycin and spectinomycin resistance	*Escherichia coli*	100	AAV69850
qacEΔ1	91893–92237	Quaternary ammonium resistance	*Escherichia coli* plasmid 1658/97	100	AAO49596
sulI	92231–93070	Sulfonamide resistance	*Escherichia coli* plasmid R100	99	NP_052895
orf116	93198–93698	Conserved hypothetical protein	*Escherichia coli* plasmid 1658/97	100	AAO49594
istB	94656–93874	Tn*21* transposition	*Shigella flexneri* Tn*21*	100	AAC33916
istA	96160–94646	IS*1326* transposase	*Klebsiella pneumoniae* plasmid RMH760	99	AAM89412
tniBΔ1	96468–96271	Transposon ATPase	*Escherichia coli* plasmid R100	99	NP_052890
tnpA	99466–96461	Tn*3* transposase	*Escherichia coli*	99	P03008
orf121	99628–100185	Tn*3* resolvase	*Escherichia coli*	100	P03011
bla_{TEM-1}	100368–101228	Beta-lactamase	*Escherichia coli*	100	AAR06285

ssb, *psiA*, *stbA*, *stbB*, *parA*, and *psiB* (13). Four replication genes, *repA1* to *repA4*, are also found on pAPEC-O2-R.

The average G+C content of pAPEC-O2-R is 53%, which is similar to that of the *E. coli* K-12 genome (4). However, several regions have notable deviations from this G+C ratio (Fig. 2). The transfer region has an average G+C content of 52%, which is markedly different from those of its flanking plasmid maintenance and gene cassette-containing regions, with G+C contents of 56% and 57%, respectively. These two regions are separated by the silver resistance operon, which has an average G+C content of 51%.

Comparative genomics. pAPEC-O2-R was compared to similar IncF plasmids whose complete sequences are available. pAPEC-O2-R was compared to *E. coli* plasmids R100 (GenBank accession no. NC_002134) and C15-1a (6), its two closest DNA sequence matches in the National Center for Biotechnology Information database. Comparison of translated coding sequences revealed that 27% of the 201 total predicted pro-

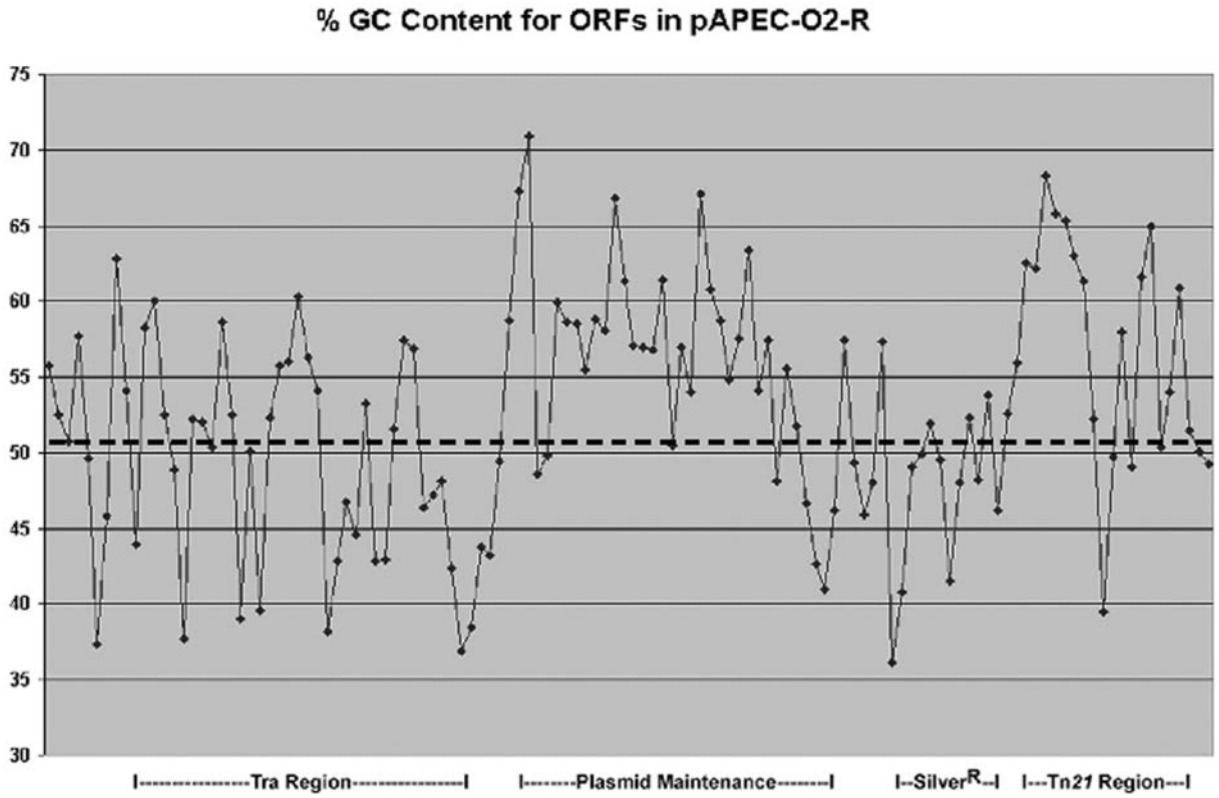

FIG. 2. Analysis of G+C contents of coding regions of pAPEC-O2-R. The dashed line represents the average G+C content of the *E. coli* K-12 genome (4).

teins were common to all three plasmids, 19% were shared by two of the three plasmids, and 54% were present in only one of the three plasmids. Most of the proteins common to the three plasmids were components of the transfer and plasmid maintenance regions of pAPEC-O2-R. By using a MAUVE alignment (10), the complete sequence of pAPEC-O2-R was aligned with the sequences of *E. coli* plasmids F (14), R100 (accession no. GenBank NC_002134), 1658/97 (accession no. GenBank NC_004998), and C15-1a (6). The alignments of these five plasmids identified a common backbone containing genes involved in plasmid transfer, maintenance, and replication. The proteins within this backbone account for approximately 40% of the total proteins within pAPEC-O2-R. The remainder of these plasmids appear to be composed primarily of antimicrobial resistance genes, mobile elements, and hypothetical proteins of unknown function.

DISCUSSION

Large plasmids are common among APEC strains and contain genes important to antimicrobial resistance (8) and virulence (12, 17, 19, 30). In this study, the first complete sequence of an APEC plasmid is presented. pAPEC-O2-R was found to contain a functional multidrug resistance-determining region, as acquisition of pAPEC-O2-R by the recipients was accompanied by acquisition of the donor strain's antimicrobial resistance pattern. This resistance region contains the *sil* gene cluster, which encodes resistance to silver and other heavy metals and which has previously been identified on large plasmids in *Salmonella* (16), *Serattia* (15), and *Klebsiella* spp. (9). Also, within this region of pAPEC-O2-R are what appear to be remnants of Tn*21*, a transposon coined the "flagship of the floating genome" for its ability to facilitate the acquisition and/or the deletion of resistance genes within the bacterial genome (24). Tn*21* has previously been identified in APEC (24). The Tn*21*-like region of pAPEC-O2-R contains an intact class 1 integron previously ascribed to Tn*21*, named In2, and the 5′ portions of Tn*21*. However, unlike the previously described structure of Tn*21* (24), the class 1 integron in pAPEC-O2-R lacks the operon encoding mercury resistance on its 3′ end. Nevertheless, the presence of a class 1 integron and other components of Tn*21* within this region of pAPEC-O2-R indicates that portions of this region might be derived from Tn*21*. The class 1 integron of pAPEC-O2-R contains three gene cassettes, including *catB3* (7), which encodes resistance to chloramphenicol; *aadA5* (33), which contributes to aminoglycoside resistance; and *folA* (1, 2), which encodes resistance to trimethoprim. All resistance genes on pAPEC-O2-R appear to be functional, as determined by disk diffusion, with the exception of the *catB3* gene encoding chloramphenicol resistance. Only an intermediate zone of inhibition was obtained when strains containing pAPEC-O2-R were grown in the presence of chloramphenicol disks. Analysis of the gene cassette region of the class 1 integron on pAPEC-O2-R identified a 132-bp *attC* site on the 3′ end of *folA*, a 60-bp *attC* site on the 3′ end of *catB3*, and a 57-bp *attC* site on the 3′ end of *aadA5*. No

promoter sequences were identified for any individual gene cassettes; only a common promoter within the *intI1* gene was identified. This class 1 integron is also flanked on its 3′ conserved end by an intact Tn*3*, which contains bla_{TEM-1}, and on its 5′ end are other remnants of Tn*21*, which is downstream of the silver resistance-determining operon.

Overall, the arrangement of the antimicrobial resistance region of pAPEC-O2-R is unique compared to that in other R plasmids. Several plasmids that encode resistance to multiple heavy metals and toxins have been sequenced, such as plasmid R478 in *Serratia marcescens* (15) and plasmid LVPK in *Klebsiella pneumoniae* (9), but they lack the class 1 integron of pAPEC-O2-R. Alternatively, several *E. coli* R plasmids that contain Tn*21*-like regions have been sequenced, such as plasmids R100 (GenBank accession no. NC_002134), C15-1a (6), and 1658/97 (GenBank accession no. NC_004998); but these plasmids lack the heavy metal resistance genes found in pAPEC-O2-R. Therefore, the composition of pAPEC-O2-R is noteworthy due to its diversity and its large number of resistance genes.

In addition to its functional multidrug resistance-encoding region, pAPEC-O2-R possesses a 31,887-bp transfer region nearly identical to that found in several *E. coli* plasmids, including the F plasmid (14) and R100 (GenBank accession no. NC_002134). This region is also similar to the transfer region of a large plasmid (pSLT) found in an *S. enterica* serovar Typhimurium strain (27). This transfer region encodes a type 4 secretion system that facilitates conjugative transfer (22). The transfer region of pAPEC-O2-R is functional, as evidenced by the fact that pAPEC-O2-R is transmissible by conjugation into commensal and pathogenic bacteria, such as *E. coli* and *S. enterica* serovar Typhimurium, that may be found in the poultry production environment. Therefore, it is possible that plasmid transfer might occur naturally in the poultry environment. Indeed, studies have shown that large plasmids are common among avian *E. coli* strains (12, 30) and that these plasmid-containing *E. coli* strains may be transmitted between birds (23). Interestingly, such transfer may also occur from birds to humans (23). In the present study, transfer of pAPEC-O2-R from APEC O2 to a human UPEC strain occurred in vitro, supporting the possibility that R plasmids harbored by animal pathogens may be reservoirs of resistance genes for human pathogens.

pAPEC-O2-R also contains genes involved in its own maintenance. Flanking the transfer region are two genes, *hok* and *sok* (for *ho*st *k*illing and *s*uppression *o*f *k*illing, respectively), involved in postsegregational killing of plasmid-free cells, thus ensuring that pAPEC-O2-R is retained during cell replication (13). Also within this region are *ssb*, *psiA*, and *psiB*, which may be involved in the conjugal transfer of pAPEC-O2-R into a recipient cell, with *psiB* inhibiting the cellular SOS response upon transfer, thus protecting the single-stranded plasmid DNA in the recipient prior to the synthesis of the second strand (25). Three more genes, *stbA*, *stbB*, and *parA*, also lie within this plasmid maintenance region and are involved in partitioning of pAPEC-O2-R into daughter cells during cell division, thus playing a role in plasmid stability (35). The presence of an active partitioning system and an antisense RNA-regulated plasmid addiction system on pAPEC-O2-R ensures that this plasmid is retained by bacterial populations, even in the absence of selective pressures within the poultry environment. Thus, these plasmids may have emerged in populations of APEC due to some type of selective pressure, such as the use of antimicrobials in the poultry environment, and they are likely retained by these APEC strains, even in the absence of this selective pressure, due to their active partitioning and plasmid addiction systems.

Additionally, pAPEC-O2-R contains four coding regions, *repA1* to *repA4*, that are likely involved in replication, copy number, and stability. BLAST analysis of these coding regions shows that they are very similar to those of IncF plasmids, a diverse group of plasmids with similar replicons and transfer regions (Table 2). The replicons included in this group are RepFIIA, whose members include pR100 and pR1; RepFIC, which is a replicon of the F plasmid; RepFIB, a replicon of ColV plasmids such as pRK100 (34); and RepFIII, a close relative of RepFII that includes *E. coli* plasmid SU316 (26). Comparison of the four predicted replication proteins in pAPEC-O2-R with those of pR100 (GenBank accession no. NC_002134), pRK100 (34), and pSU316 (26) revealed that pAPEC-O2-R shares the strongest identity with pR100, an IncFII plasmid. The *repA1*-coding sequence, which is directly involved in plasmid replication, and *repA4*, a gene immediately adjacent to the origin of replication that is involved in plasmid stability (18), appear to be highly conserved (99% protein identity). The *repA2*- and *repA3*-coding sequences, which are involved in replication control, were quite different among the four plasmids analyzed, exhibiting only partial protein identity to published sequences (Table 2). Others have also reported that these portions of IncF replicons are areas of nonhomology (26). However, these coding regions in pAPEC-O2-R are considerably different from any sequences published to date. Further work is required to determine the significance of these differences.

In summary, a 101-kb IncF plasmid from an APEC strain was sequenced and analyzed, providing the first completed APEC plasmid sequence. This plasmid, pAPEC-O2-R, contains genes for plasmid maintenance and replication. It also has a functional transfer region that allows its transmission to bacterial strains that are found in the poultry environment or that cause human infection. Additionally, pAPEC-O2-R contains an antimicrobial resistance-encoding region that encodes multidrug resistance. This region of the plasmid is unique among previously described IncF plasmids, as it possesses a class 1 integron that harbors three gene cassettes and a heavy metal resistance operon. Differences in the G+C contents of individual ORFs suggest that various regions of pAPEC-O2-R had dissimilar origins. The presence of pAPEC-O2-R-like plasmids that encode resistance to multiple antimicrobial agents and that are readily transmissible suggests the possibility that such plasmids may serve as a reservoir of resistance genes for other bacteria of animal and human health importance.

ACKNOWLEDGMENT

This project was funded in part by the Roy J. Carver Charitable Trust Fund.

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