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Identification of Avian Pathogenic *Escherichia coli* Genes That Are Induced *In Vivo* during Infection in Chickens

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Avian pathogenic *Escherichia coli* (APEC) is associated with extraintestinal infections in poultry causing a variety of diseases collectively known as colibacillosis. The host and bacterial factors influencing and/or responsible for carriage and systemic translocation of APEC inside the host are poorly understood. Identification of such factors could help in the understanding of its pathogenesis and in the subsequent development of control strategies. Recombination-based *in vivo* expression technology (RIVET) was used to identify APEC genes specifically expressed during infection in chickens. A total of 21 clones with *in vivo*-induced promoters were isolated from chicken livers and spleens, indicative of systemic infection. DNA sequencing of the cloned fragments revealed that 12 of the genes were conserved *E. coli* genes (*metH*, *lysA*, *pntA*, *purL*, *serS*, *ybjE*, *ycdK* [*rutC*], *wcaJ*, *gspL*, *sdsR*, *ylbE*, and *yjiY*), 6 of the genes were phage related/associated, and 3 genes were pathogen specific (*tkt1*, *irp2*, and *eitD*). These genes are involved in various cellular functions, such as metabolism, cell envelope and integrity, transport systems, and virulence. Others were phage related or have yet-unknown functions.

Avian pathogenic *Escherichia coli* (APEC) is associated with extraintestinal infections in chickens, turkeys, ducks, and other avian species, causing a variety of diseases collectively known as colibacillosis. The infections are responsible for severe economic losses in the poultry industry worldwide (7, 25, 29). A number of virulence factors have been identified as associated with APEC infections (23, 29, 43, 83), some of which have been shown to contribute to pathogenicity. They include autotransporter genes (28, 54), iron uptake systems (13, 14, 36, 75), lipopolysaccharide (LPS) O antigens (66), K1 capsule (66), certain fimbrial adhesins (21, 50), and secretion systems (24). However, the mechanisms underlying pathogenicity are still poorly understood (29). The availability of the complete genome sequence of APEC O1:K1:H7 in combination with experimental infection models in avian hosts provides the basis for comprehensive understanding of APEC pathogenesis (4, 42).

With genome-wide analyses in recent years, several new molecular approaches have provided a better understanding of the molecular mechanisms of pathogenicity of microorganisms (35, 60, 89). Recently, techniques such as selective capture of transcribed sequences (SCOTS), signature-tagged mutagenesis (STM), genomic suppression subtractive hybridization (GSSH), and transcriptome analysis using microarrays were applied to APEC to identify different genes associated with virulence and/or pathogenicity in chickens (27, 55, 56, 81). Although some of these techniques provide evidence that certain genes may contribute to virulence, it is of interest to determine which genes are actually expressed *in vivo*, as they may be required for APEC to cause infection and could be potential vaccine candidates (16). Identification of differentially active promoters *in vivo* and, if possible, those for putative and unknown virulence genes would provide more information on pathogenesis.

One of the methods that has been used successfully in bacteria to study the promoters that are specifically active *in vivo* and not *in vitro* is *in vivo* expression technology (IVET) (60, 61). Recombination-based *in vivo* expression technology (RIVET) is a variant of the original *in vivo* expression technology that uses a promoterless site-specific recombinase and a pair of recombinase target sequences flanking a selectable marker. When active promoters are fused to the promoterless recombinase, induction of recombinase expression occurs. This subsequently causes recombination of the two target sequences, deleting the intervening marker, and permanently changes the bacterial phenotype that can be detected after gene expression has ceased (10, 74). RIVET has been used in several Gram-negative and Gram-positive bacteria to identify promoters and/or genes that are induced *in vivo* (5, 9, 11, 12, 33, 38, 58, 80, 86, 91).

In this study, the use of RIVET for the identification of APEC promoters and/or genes induced specifically *in vivo* during infection in chickens is described, and the findings suggest that there is a range of genes that are induced as a response of the pathogen to the new environment.

MATERIALS AND METHODS

Bacterial strains, plasmids, media, and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. APEC strain CH2 (O78) was isolated from an infected chicken and is virulent in experimentally infected chickens (90). Strains containing plasmids pKD46 (22) and pSC101-BAD-Cre-tet (3) were incubated at 30°C unless otherwise indicated. Those containing plasmid pR6K-*rpsL*-Neo^r (34) were incubated at 37°C. Luria-Bertani (LB) medium and SOC medium were prepared as described elsewhere (76). All bacteria were routinely grown in LB broth or agar medium at 37°C unless otherwise indicated. Antibiotics for plasmid and/or recombinant selection were added at the following concentrations: ampicillin (Amp), 100 μg ml^{-1}; kanamycin (Kan), 50 μg

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TABLE 1 Bacteria and plasmids used in this study

Strain or plasmid	Relevant feature(s)	Reference
Strains		
APEC CH2	O78 serotype isolated from infected chicken	90
APEC CH2-Str^R	Streptomycin-resistant derivative of APEC CH2	This study
APEC CH2-Str^R/pKD46	APEC CH2-Str^r derivative containing pKD46 (*oriR101 bla* P_{BAD}-λ *gam bet exo*)	This study
CH2LoxP	APEC CH2-Str^r derivative containing *loxP-rpsL-neo-loxP* cassette in the *fiu* gene	This study
Plasmids		
pFLAG-MAC	Cytoplasmic expression vector	Sigma
pSC101-BAD-Cre-tet	Cre expression vector under the promoter P_{BAD}	3
pR6K-rspL-neo	R6K gamma *ori*, *rpsL-neo* cassette	34
pHT001	Derivative of pFLAG-MAC	This study
pHT002	pHT001 containing promoterless *cre*	This study
pHT003	pHT002 containing *E. coli lpp* promoter	This study

ml^{-1}; streptomycin (Str), 100 μg ml^{-1}; and tetracycline (Tet), 5 μg ml^{-1}. A streptomycin-resistant (Str^r) derivative of APEC CH2, designated CH2-Str^r, was obtained by serial culturing in increasing concentrations of streptomycin, as previously described (88). *E. coli* strain Top10F′ (Invitrogen, Paisley, Scotland) was used as the cloning strain.

DNA manipulation. DNA manipulations were performed as described elsewhere (76). Electrocompetent cells were prepared using standard procedures unless otherwise indicated (76). Electroporation was carried out as previously described (88). CH2 genomic DNA, plasmid DNA, and DNA fragments were purified using commercial kits purchased from Fermentas (St. Leon-Rot, Germany). DNA restriction and modification enzymes were purchased from Fermentas and used as recommended by the manufacturer. PCR amplifications were performed using AccuPrime *Taq* High Fidelity polymerase (Invitrogen) or SuperTaq polymerase (SphaeroQ, Leiden, The Netherlands). Oligonucleotides were ordered from Sigma-Aldrich (Bornem, Belgium) (Table 2).

Construction of the RIVET host strain. An APEC CH2-Str^r strain harboring a chromosomally located *loxP-rpsL-neo-loxP* cassette was constructed as previously described for the strain APEC 1-Str^r (88). Briefly, APEC CH2-Str^r strains were made electrocompetent and transformed with plasmid pKD46 encoding the lambda Red recombinase by electroporation. For the integration of the *loxP* cassette, the lambda Red recombineering technique (22) was used, whereby APEC CH2-Str^r containing pKD46 was made electrocompetent and approximately 0.1 μg to 0.3 μg of PCR products (amplified using primers HT53F and HT53R) containing the *loxP-rpsL-neo-loxP* cassette (LoxP cassette) flanked by regions homologous to the *fiu* gene was electroporated. The kanamycin-resistant (Kan^r) colonies obtained were purified selectively at 37°C and then grown at 43°C to cure the plasmid pKD46. The strain was designated CH2LoxP.

Construction of a vector containing promoterless *cre*. The plasmid pFLAG-MAC (5.1 kb; Sigma) was digested by EcoRV to remove the FLAG coding sequence, part of the *lacI* gene, and the ribosomal binding site (RBS) and recircularized to form plasmid pHT001 (3.7 kb). An EcoRI/XhoI restriction fragment from plasmid pSC101-BAD-Cre-tet containing a promoterless Cre recombinase gene (*cre*) was cloned into pHT001 digested with EcoRI/XhoI to form pHT002 (4.7 kb) (Fig. 1A). PCR amplification, using primers HT86F and HT86R flanking the cloning site, was performed to verify the proper insertion of the fragment containing the *cre* gene.

Construction of an APEC CH2 RIVET library. The APEC CH2 RIVET library was made by partial digestion of APEC CH2 genomic DNA with Sau3AI, size fractionating it on a 1% agarose gel, and cloning the fragments (0.5 to 2 kb) into the BglII site of pHT002. The ligation mixture was transformed into *E. coli* Top10F′ (Invitrogen). The colonies obtained (approximately 60,000) were pooled, and the plasmid DNA was isolated from the cells. Random clones were picked, and the average insert size was determined. The plasmid DNA was transformed into CH2LoxP strains and plated on LB with Amp and Kan. Approximately 28,000 colonies of the APEC RIVET library were collectively resuspended in LB containing 15% glycerol and stored in aliquots at −80°C.

Screening of the APEC CH2 RIVET library for *in vivo*-induced genes. Animal studies were approved by the Ethical Committee for Animal Experiments of the Katholieke Universiteit Leuven according to international regulations (project number P119/2009). Approximately 1×10^9 CFU from the APEC RIVET library was administered intratracheally using a feeding needle (malleable metal, silicone tip, sterile, plastic Luer hub, 20 gauge; AgnTho's AB, Lidingo, Sweden) into four 3-week-old broiler chickens (Ross; Belgabroed NV, Merksplas, Belgium), which were housed in a disinfected stable and supplied with water and feed *ad libitum*. After 24 h, the chickens were sacrificed by cervical dislocation, and their livers and spleens were collected aseptically and homogenized in sterile phosphate-buffered saline (PBS) (1 g of tissue per 1 ml sterile PBS). The dilutions were plated on MacConkey agar with Amp. After incubation at 37°C for 24 h, the colonies obtained were then replica plated on MacConkey agar with Amp

TABLE 2 Oligonucleotides used in this study

Name	Sequence (5′–3′)	Reference
HT53F[a]	CCCGATACCCCAGTTTGGCGTCGGCGACCCAATAACCTTCGGTAAATGCTGGCGTCCCCACCGCGCCGTC **ataacttcgtatagcatacattatacgaagttat***GGCCTGGTGATGATGGCGGGATCG*	88
HT53R[a]	CCGAAATTCTCGCGTCCGGTAGCGGATACTACCCGCACGATGACGGTGATTTCTGAACAAGTGATTAAAG **ataacttcgtataatgtatgctatacgaagttat***TCAGAAGAACTCGTCAAGAAGGCG*	88
HT57F	TGGCTGTGAGCAAGAAGGTTCTTGGC	88
HT57R	ACGCGGATGACACGCTGGTTGT	88
HT86 F	CTACAAGGACGACGATGACAA	This study
HT86 R	TCAGGCTGAAAATCTTCTCTC	This study
CRESEQ	CATTTTCCAGGTATGCTCAG	38

[a] Primer used to isolate the *rpsL-neo* cassette from plasmid pR6K-rpsL-neo. From the 5′ end, 70 bp are homologs of the target region; the lowercase boldface letters are *loxP* sites, while the italicized letters represent priming sites to the template plasmid pR6K-rpsL-neo.

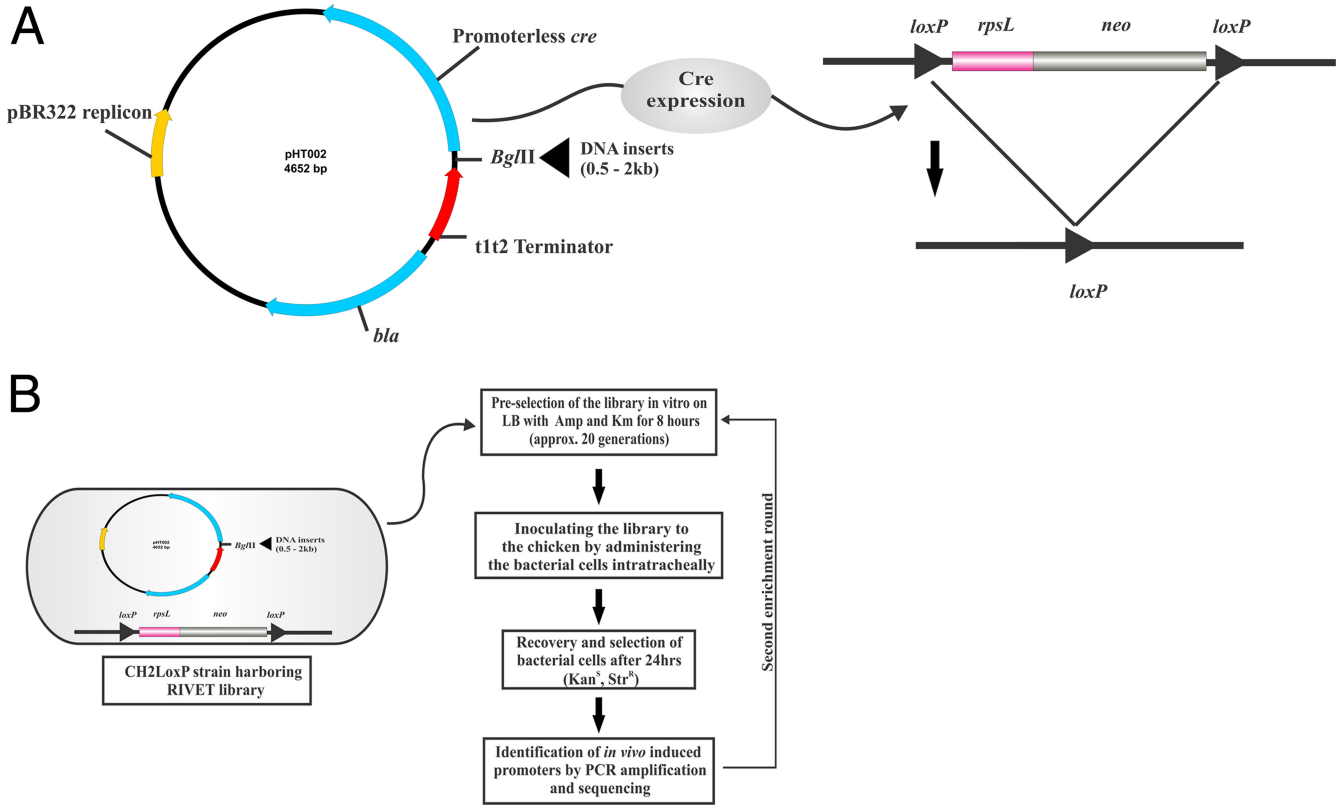

FIG 1 RIVET strategy for use in APEC to identify promoters that are induced *in vivo*. (A) An *rpsL-neo* cassette flanked by two copies of site-specific recombination sequences (*loxP*) was integrated into the APEC CH2 Str^r chromosome. An APEC CH2 promoter trap library was constructed by cloning random DNA fragments in plasmid pHT002 upstream from a promoterless *cre* gene. If an active promoter is thus cloned, the recombinase Cre is expressed, resulting in a homologous recombination of the *loxP* sites. This results in the irreversible excision of the *rpsL-neo* cassette. (B) The APEC CH2 promoter trap library (RIVET library) was transformed into the CH2LoxP strain. After preselection on kanamycin to eliminate bacterial cells containing active promoters under *in vitro* conditions, the resulting library was administered intratracheally into a chicken. Irreversible changes in the antibiotic resistance phenotype of the CH2LoxP strain allowed selection of resolved bacteria containing *in vivo*-induced promoters. After isolation of the bacteria, the plasmids contacting *in vivo*-induced promoters were subjected to a second enrichment round.

and Str and incubated again at 37°C for 24 h. The bacteria that had lost the LoxP cassette due to differential expression of *cre* regained Str resistance and hence grew in this medium. The loss of the LoxP cassette was further verified by Kan sensitivity (Kan^s) and colony PCR using primers HT57F and HT57R. The colonies obtained on MacConkey agar with Amp and Str were picked up, and plasmids were isolated and purified for sequencing of the inserts using primers CRESEQ and HT86R, annealing in *cre* and upstream from the inserted DNA fragment, respectively, on pHT002. The sequences were analyzed using BLASTX (http://www.ncbi.nih.gov) and compared to the sequences available in GenBank.

Nucleotide sequence accession numbers. The sequences of the selected 21 unique clones were submitted to GenBank with accession numbers JQ582670, JQ582671, JQ582672, JQ582673, JQ582674, JQ582675, JQ582676, JQ582677, JQ582678, JQ582679, JQ582680, JQ582681, JQ582682, JQ582683, JQ582684, JQ582685, JQ582686, JQ582687, JQ582688, JQ582689, and JQ582690.

RESULTS

Adaptation of RIVET screening for APEC. The general scheme for RIVET is shown in Fig. 1A. The Cre-*lox* system was shown to be functional in APEC strains (88), which suggests that the Cre recombinase is suitable for the construction of a RIVET screening system in this bacterium. The reporter strain CH2LoxP was constructed by integrating a LoxP cassette in the chromosomally located *fiu* gene, one of the 12 iron receptor genes in APEC encoding an iron-regulated outer membrane protein known as ferric iron uptake protein (19, 68, 69). The LoxP cassette contains a floxed *rpsL-neo* marker where *rpsL* is used for positive selection of colonies with an active promoter while *neo* is used for negative selection. Confirmation of integration was done using primers flanking the *fiu* gene and locus-specific primers as described previously for the APEC1LoxP strain (88). There were no significant differences between CH2LoxP and APEC CH2 in terms of *in vitro* growth for 12 h or survival *in vivo* inside the host for 48 h (Fig. 2A and B). The stability of integration of the LoxP cassette was confirmed *in vitro* and *in vivo*. The CH2LoxP strain was still Kan^r after growth without antibiotic selection pressure for 96 h with 4 subculturing steps in between, followed by plating on LB-Kan. *In vivo*, Kan^r colonies could be isolated 48 h after infection of the chicken host with CH2LoxP. PCR analysis confirmed that all selected colonies contained an intact LoxP cassette (data not shown). The whole LoxP cassette was also sequenced to confirm the orientation of the *loxP* sites. Taken together, these experiments indicate that the cassette was stably integrated into CH2LoxP and maintained during infection in chickens and that this did not affect its virulence or growth under standard laboratory conditions.

In the next step, the promoter trap plasmid pHT002, containing a promoterless *cre* gene and a pBR322 origin of replication,

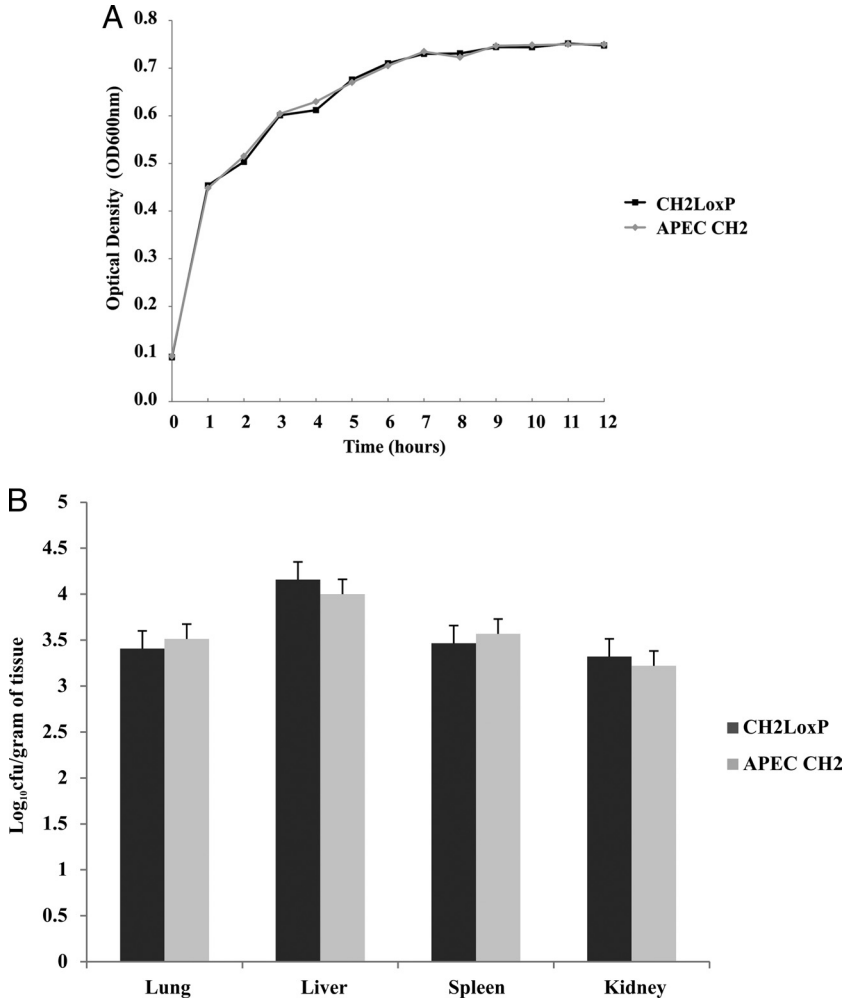

FIG 2 Comparison of growth and survival of APEC CH2 and its isogenic mutant CH2LoxP strain. (A) Growth comparison of the two strains *in vitro*. There were no significant differences in terms of growth in LB medium after growing the strains separately for 12 h. (B) The *in vivo* survival of the strains was evaluated. The strains were separately administered intratracheally into chickens. After 48 h, the bacteria were isolated from the lungs, livers, spleens, and kidneys of four different chickens. There were no significant differences between the two strains in terms of CFU counts from organs.

was created. It also contained a *bla* gene for Amp resistance, an *E. coli* terminator to prevent transcriptional readthrough, and a BglII site for the insertion of the APEC CH2 genomic library. As a positive control, a derivative of pHT002 was made by cloning a strong *E. coli* constitutive promoter from the *lpp* gene encoding *E. coli* lipoprotein (40) at the BglII site and designated pHT003. Plasmid pHT002 and its derivative, pHT003, were introduced into the reporter strain CH2LoxP to evaluate their functionality. The strains were plated on LB-Amp and LB agar plates with Amp and Kan and with Amp and Str. After 48 h of growth, none of the pHT002 transformants became Str^r while more than 98% of the pHT003 transformants became Str^r, suggesting excision of the *rpsL* marker (data not shown). PCR using the HT57 primer pair was performed on CH2LoxP containing pHT003 from LB agar plates with Amp and confirmed that the LoxP cassette was indeed deleted. These results confirm the suitability of the reporter strain CH2LoxP, together with the promoter trap plasmid pHT002, for the RIVET application.

Construction of the APEC CH2 RIVET library in pHT002. A genomic library of APEC CH2 was constructed by cloning random genomic DNA fragments of CH2 (0.5 kb to 2 kb) into the BglII site of pHT002. The *E. coli* Top10F′ strain was used as an intermediate cloning host. A total of approximately 28,000 colonies were obtained in the CH2LoxP strain (RIVET library) after preselecting the strains on LB agar plates with Amp and Kan. This selection excluded *in vitro* active promoters, which would induce the expression of *cre* and subsequently cause the deletion of the *loxP* cassette, making the strain Kan^s. Insert analysis of the library was performed by PCR using primers CRESEQ and HT86R (Table 2) on 90 randomly picked clones containing pHT002 derivatives, and it revealed the estimated average size of the insert to be approximately 1 kb (data not shown). To evaluate the library redundancy, 27 inserts were sequenced, suggesting that the library was random and not redundant. The genome coverage was estimated using the formula of Clarke and Carbon (18) and found to be higher than 99%.

RIVET screen in the chicken host model. The RIVET screening approach is depicted in Fig. 1B. The library was grown for 8 h in the presence of Amp and Kan to counterselect against clones displaying *cre* expression *in vitro*. The cultures grown were used

TABLE 3 Functions and features of the APEC CH2 genes induced during infection in chickens

Gene function of *in vivo*-induced clone	No. of clones isolated	Orf/gene	Similar gene(s) in GenBank		
			Accession no.	% Identity	Putative gene function and/or feature
Metabolism	23	*metH*	ACD10039.1	93	5-Methyltetrahydrofolate-homocysteine methyltransferase
	39	*lysA*	YP_854078.1	100	Diaminopimelate decarboxylase
	37	*pntA*	EFQ02994.1	100	NAD(P) transhydrogenase alpha subunit
	23	*purL*	EGW90584.1	99	Phosphoribosylformylglycinamidine synthase
	24	*serS*	EGK39589.1	100	Seryl-tRNA synthase
	47	*tkt1*	YP_859417.1	100	Transketolase
	25	*ybjE*	YP_851966.1	99	L-Lysine exporter
	25	*rutC* (*ycdK*)	YP_540087.1	99	Pyrimidine catabolism
Cell envelope and integrity	29	*wcaJ*	EHN96105.1	97	Putative colanic acid biosynthesis UDP-glucose lipid carrier
Transport systems	30	*gspL*	EFU48119.1	99	Putative type II secretion protein GspL
	26	*sdsR*	EFW75368.1	97	Putative membrane fusion protein of efflux pump
Virulence	28	*irp2*	EEH86983.1	98	Yersiniabactin biosynthesis protein
	22	*eitD*	YP_444144.1	93	Putative iron transport system, membrane component
Phage related	27		EGE65170.1	99	Putative minor capsid protein GPC
	26		EHN80486.1	99	Putative tail component of prophage
	26		EGU96028.1	100	Putative replication protein
	33		YP_853767.1	90	Bacteriophage V tail/DNA circulation protein
	31		ZP_08376451.1	99	Host specificity protein
	29		ZP_03357336.1	93	Putative bacteriophage tail protein
Unknown function	29	*yjiY*	EGB64734.1	99	Putative carbon starvation family protein
	30	*ylbE*	ACI87286.1	99	Conserved hypothetical protein

for intratracheal administration to chickens. To identify genes important for systemic infection, approximately 4,000 clones were recovered from livers and spleens after plating on MacConkey agar with Amp. Replica plating on MacConkey agar with Amp and Str resulted in 139 Str^r clones (approximately 3.5%). To verify the primary RIVET results, plasmids representing these 139 clones were isolated, purified, and reintroduced individually by electroporation into the original CH2LoxP strain (Kan^r Str^s). The resulting colonies were Amp^r and Kan^r, confirming the absence of *cre* expression in these clones when grown *in vitro*. The clones were pooled and subjected to a second round of infection in chickens and screening, as explained above, to identify only promoters that showed reproducible induction in different chickens. Out of 1,320 colonies obtained after plating on MacConkey agar with Amp, 630 colonies were Amp^r and Str^r (approximately 48%). These clones were subjected to plasmid isolation, purification, and sequencing of the insert in the plasmids. After performing a BLASTX search, the screening resulted in 21 unique clones, of which 12 genes were *E. coli* conserved genes, 6 were bacteriophage associated, and 3 (*tkt1*, *irp2*, and *eitD*) were pathogen-specific genes. The number of clones isolated per gene is shown in Table 3. Various cellular functions, such as metabolism, adaptation, stress response, cell membrane and integrity, transport systems, virulence, and phage-related or unknown functions, were assigned to the identified genes (Table 3) based on the highest percent identity/similarity with the sequences available in GenBank.

DISCUSSION

Bacterial pathogens such as APEC encounter unique environments inside the host during infection, so the pathogen has to respond by the coordinated expression of regulatory, metabolic, and virulence genes (30, 64). As it has been suggested that genes induced during infection can play a critical role in virulence (64, 70), *in vivo* experimental models that allow the recognition of bacterial genes that are expressed during infection are highly advantageous (53). Recently, studies using SCOTS and STM have been used to identify APEC host-induced genes with the goal of finding candidate colonization and disease-promoting factors (27, 55). In this study, RIVET using the Cre/*lox* system was developed and used for the first time to investigate APEC genes that are host induced *in vivo* during infection in chickens. The major advantages of RIVET over other strategies include the detection of transient gene induction in a small number of cells. RIVET also allows identification of niche-regulated genes expressed at different levels (11, 70). The disadvantages of SCOTS, such as the instability of bacterial mRNA for the construction of cDNA libraries, the low abundance of mRNA from transiently expressed genes, and the difficulty in isolation of sufficient high-quality mRNA from small populations of bacteria *in vivo*, do not apply to IVET and RIVET (59). In the STM approach, only limited numbers of mutants can be screened per animal model, and moreover, mutants that are slow growing or nonviable, that contain mutations in genes encoding redundant functions, or that can be complemented in a mixed population may be missed during screening. These disadvantages do not apply to RIVET (59).

In this study, four genes were identified as being involved in amino acid metabolism. They are *metH*, which encodes cobalamin-dependent methionine synthase (6); *serS*, which encodes seryl-tRNA synthase (20); *lysA*, which encodes diaminopimelate decarboxylase (85); and *ybjE*, which encodes an L-lysine exporter (67). *serS* and *lysA* were previously found to be induced *in vivo* in other pathogens, demonstrating the effectiveness of the system used in this study (31, 52). Moreover, *lysA* was found to be important for the virulence of *Staphylococcus aureus* in a murine model of bacteremia (63). Other *in vivo* approaches have shown that genes involved in amino acid metabolism in *Vibrio cholerae* (11), *E. coli* (48), and *Helicobacter pylori* (12, 47) were induced during

mouse infection. The nucleic acid biosynthesis gene *purL*, which encoded 5′-phosphoribosylformylglycinamide amidotransferase, was identified. The enzyme catalyzes the conversion of 5′-phosphoribosylformylglycinamide to formylglycinamidine in the presence of glutamine and ATP for *de novo* purine nucleotide biosynthesis (77). The induction of the genes related to amino acid metabolism and nucleic acid biosynthesis could indicate that the requirement for these molecules is high for the survival of the bacteria in the hostile environment inside the chicken host. This was also the case for uropathogenic *E. coli* (UPEC), which required gluconeogenesis and the tricarboxylic acid (TCA) cycle during urinary tract infection in mice, as the peptides and amino acids were shown to be the primary carbon sources (2). The *pntA* gene encoding the pyridine nucleotide transhydrogenase alpha subunit, which plays a major role in the production of NADPH (17, 79), was also induced in our APEC strain, indicating the importance of the production of reducing power in the cell for energy metabolism. Previously, the *pntB* gene encoding the pyridine nucleotide transhydrogenase beta subunit was identified as being induced in *E. coli* strain i484 *in vivo* during mouse infection (48). In addition, the *ycdK* (now called *rutC*) gene was identified as being induced *in vivo*. This gene has been shown to be part of a system involved in pyrimidine catabolism and is under the control of the nitrogen starvation regulator *ntrC*. A *rutC* insertion mutant lost the ability to utilize pyrimidine nucleosides and bases as the sole source of nitrogen at room temperature (57). The *in vivo* induction of this gene by APEC is probably due to a nitrogen-limited environment inside the avian host and the metabolism of nucleobases, as was shown in the case of UPEC (1).

The *tkt1* homolog represents a gene encoding a transketolase enzyme (42). Generally, transketolases catalyze the reversible transfer of a ketol group in the pentose phosphate pathway (45, 46), responsible for production of essential cell constituents, such as amino acids, NADPH, and several sugar phosphate intermediates (39, 93). The pathway is also believed to play a protective role during oxidative stress by the production of reducing power via NADPH (49, 72). The gene is found in one of the genomic islands in extraintestinal *E. coli* (ExPEC) and shares 68% and 69% identity with the *tktA* and *tktB* genes, respectively, which are also present in ExPEC and *E. coli* K-12 (42, 93). A recent study demonstrated that the TktA protein is involved in antibiotic and oxidative stress through interaction with MarR, the repressor of the operon *marRAB*, conferring resistance to multiple antibiotic and oxidative stresses on *E. coli* (26). This, together with the production of reducing power for energy metabolism, could be the reason why a *tkt1* homolog was induced *in vivo* in the chicken host for survival and colonization of APEC. In previous studies, it was shown that the *tktB* gene was upregulated when APEC was grown in chicken serum (56). Moreover, APEC could not survive inside the chicken when the *tktA* gene was knocked out, showing the importance of transketolase enzymes in APEC (55).

In addition, another gene that is involved in stress response after encountering the host environment was identified. The gene, known as *sdsR* (*yjcR*), is found in the operon *sdsRQP* encoding a putative multidrug efflux pump involved in sensitivity control against sulfur drugs (82). The gene is under the control of the LeuO protein, a global regulator controlling a number of genes, including stress response genes and virulence-related genes. Due to stresses, such as upon entry into stationary phase, increased expression of LeuO was observed in several *E. coli* strains, which also activated the operon *sdsRQP* (82).

The only gene identified as being associated with the cell envelope and integrity is *wcaJ*. The gene encodes a putative UDP glucose lipid carrier transferase in the biosynthesis of colanic acid (CA). CA is one of the exopolysaccharides (EPS) that are produced by *E. coli*, forming a thick mucoid matrix on the cell surface (84). It was demonstrated that a CA-deficient mutant of *E. coli* O157:H7 was less tolerant of acid, heat, osmotic, and oxidative stresses (15, 62). Also, an APEC *wcaE* mutant was unable to survive inside chickens after infection (55). As *wcaJ* was identified in this APEC strain as *in vivo* induced, this suggests that CA is produced to confer protection against host environmental stresses, such as the body temperature of chickens (42°C) and the acid environment in macrophages, where it was demonstrated that APEC O1:K1:H7 could survive intracellularly (73). Moreover, using an STM approach, an APEC *wcaE* mutant was shown to be attenuated *in vivo* (55), further confirming the importance of CA for APEC survival *in vivo*.

The *gspL* gene encodes one of the proteins of the putative type II secretion system (T2SS) (GspL-like protein). In the sequenced APEC O1 chromosome, it is located in pathogenicity island I (PAI $I_{APEC\ O1}$) (42). The T2SS, also known as the general secretory pathway (GSP), is one of the systems used by most Gram-negative bacteria to transport folded proteins, including toxins, proteases, cellulases, and lipases, from the periplasm to the extracellular medium through the outer membrane channel (44). Induction of this gene indicates that the operon coding for the T2SS could be active and induced in the chicken host. APEC might use the T2SS to secrete proteins responsible for pathogenesis or its fitness inside the chicken host. In UPEC, it was actually shown by mutant analysis that the T2SS is important for persistence in the urinary tract, particularly in renal tissues (51). Also, it was shown that this system is responsible for the translocation of a variety of proteins that mediate pathogenic effects, including the pore-forming toxin aerolysin of *Aeromonas hydrophila* (37), the cholera toxin of *V. cholerae* (78), and the heat-labile (LT) toxin of enterotoxigenic *E. coli* (ETEC) (87). Recently, transcriptome analysis of APEC O1 indicated that the T2SS was significantly downregulated when the bacteria were grown in chicken serum compared to growth in LB. This contrasting finding could be due to the fact that the analysis was limited to serum (56) or due to the heterogeneity of the APEC strains used in the different studies. Furthermore, it might be due to the fact that *gspL* is only transiently induced in chickens. Our RIVET screen could have detected the induction of *gspL in vivo*, as it can detect even transient gene induction leaving irreversible phenotypic change to the bacteria inside the host.

The *irp2* gene encodes high-molecular-weight protein 2 (HMWP2), which is involved in yersiniabactin (siderophore) biosynthesis and is located in the *Yersinia* high-pathogenicity island (HPI) in APEC O1 (32, 42). The HPI was shown to be involved in the virulence of *Yersinia pestis*, *Yersinia enterocolitica*, and *Yersinia pseudotuberculosis* and APEC O1 (56, 71). The available free iron in host tissue is, significantly, in low concentrations, forcing APEC to acquire iron from the host. The induction of this gene *in vivo* indicates that iron acquisition systems are required for APEC survival in the host. It was also demonstrated in a recent study on APEC O1 that genes involved in iron uptake were among the significantly upregulated genes (56). It was hypothesized that the increased virulence of HPI-positive *Enterobacteriaceae*, including

APEC, is caused by yersiniabactin, which not only provides iron to the bacterium, which is normally bound to iron-binding proteins produced by hosts, but also prevents reactive oxygen species (ROS) production by innate immune cells, thus promoting the survival of the bacteria in the host (71). Another *in vivo*-induced gene in connection with iron sequestration systems found in this study is *eitD*. This gene is located in the *eitABCD* operon encoding putative iron transport systems identified first on a ColV plasmid of an APEC O2 strain (43), then on a ColBM plasmid of an APEC O1 strain (41), and lately on pAPEC-2 of APEC_7122 (65).

Six phage-related genes were also identified in this study as *in vivo* induced (Table 3). APEC is believed to carry prophages in its genome (42), which under stress conditions, such as H_2O_2 and the reactive oxygen species generated and released by leukocytes, may trigger the induction of these prophages inside the host (92). Previously, a study utilizing a SCOTS approach demonstrated that APEC bacteriophage-related genes were expressed *in vivo* (27). Also, in another study with *Streptococcus mitis*, it was demonstrated that adhesion of the pathogen to the platelets was phage mediated, where genes for two surface proteins responsible for adhesion (PblA and PblB) resembled phage capsid and tail fiber genes and were surrounded by other phage-like gene sequences (8). This indicates that bacteriophage-related genes coding for virulence properties, such as toxins and adhesins, may also be induced during APEC infection, although it is not yet determined whether these phage-encoded genes are associated with the virulence of the APEC CH2 strain.

Finally, genes with unknown functions were also identified in this study. Two genes, *ylbE*, encoding a conserved hypothetical protein, and *yjiY*, which is a paralog of *cstA*, encoding a peptide tranporter induced during carbon starvation (8, 83), were induced. The fact that these genes are induced *in vivo* is an interesting observation in the further analysis of their functions.

In conclusion, the fact that we could identify host-induced genes that were previously identified in other studies validates the RIVET approach. However, there appears to be little overlap between the APEC genes identified in the STM study (55) and by SCOTS (27) and those identified by this RIVET screen. This comparison further demonstrates that STM, SCOTS, and RIVET are complementary, and not redundant, functional-genomics screens. This study provides a highlight on the APEC genes that are induced inside the chicken host during infection and that are likely to be involved in the survival and successful colonization of the pathogen in the stressful host environment. To our knowledge, this is the first report to use RIVET in this pathogen. These genes may be required for APEC pathogenesis and could be potential vaccine candidates (16). However, further experiments, such as mutational analysis, should confirm the requirement for these genes by the pathogen during infection in the chicken. Other techniques, such as quantitative real-time PCR or microarray analyses, will be required to confirm whether these conserved *E. coli* genes are actually expressed at high levels *in vivo* in infected tissues or during growth *in vitro*.

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