Transcriptome Analysis of Avian Pathogenic *Escherichia coli* O1 in Chicken Serum Reveals Adaptive Responses to Systemic Infection[▽]

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Infections of avian pathogenic *Escherichia coli* (APEC) result in annual multimillion-dollar losses to the poultry industry. Despite this, little is known about the mechanisms by which APEC survives and grows in the bloodstream. Thus, the aim of this study was to identify molecular mechanisms enabling APEC to survive and grow in this critical host environment. To do so, we compared the transcriptome of APEC O1 during growth in Luria-Bertani broth and chicken serum. Several categories of genes, predicted to contribute to adaptation and growth in the avian host, were identified. These included several known virulence genes and genes involved in adaptive metabolism, protein transport, biosynthesis pathways, stress resistance, and virulence regulation. Several genes with unknown function, which were localized to pathogenicity islands or APEC O1's large virulence plasmid, pAPEC-O1-ColBM, were also identified, suggesting that they too contribute to survival in serum. The significantly upregulated genes *dnaK*, *dnaJ*, *phoP*, and *ybtA* were subsequently subjected to mutational analysis to confirm their role in conferring a competitive advantage during infection. This genome-wide analysis provides novel insight into processes that are important to the pathogenesis of APEC O1.

Avian colibacillosis is one of the most significant infectious diseases in the poultry industry, resulting in annual multimillion-dollar losses due to mortality, lost production, and condemnations (5). Avian pathogenic *Escherichia coli* (APEC) infections take many forms, with one of the more common being septicemia (5). In order to cause septicemia, APEC must evade a multitude of host defense mechanisms in the bloodstream, including the bactericidal effects of phagocytosis, serum complement, and antimicrobial peptides (38). Indeed, serum resistance has been shown to be an important virulence trait of APEC (37). Previous studies have shown that many bacterial traits contribute to *E. coli*'s serum resistance, including production of a capsule, a smooth lipopolysaccharide (LPS) layer, and certain outer membrane proteins (OMPs) (37, 55), including OmpA, TraT, and Iss (11, 40, 42, 59). OmpA is a major protein in *E. coli*'s outer membrane, TraT is encoded by conjugative F plasmids, and Iss is homologous to the Bor protein of bacteriophage λ , which is typically encoded by large virulence plasmids in APEC (29). Such OMPs may contribute to APEC's serum resistance by re-

stricting C3 deposition on the bacterial surface (43), blockage of the membrane attack complex (31), or some other as yet unknown mechanism.

However, merely resisting the bactericidal effects of serum is not sufficient to allow APEC to proliferate in serum; APEC must also adjust its metabolism to suit the nutrients available in serum. For example, the concentration of free iron in animal serum is extremely low because iron is sequestered by host compounds like transferrin. Since iron is necessary for a series of bacterial functions, APEC has developed several strategies to acquire iron in such iron-limiting environments. At least five iron uptake and assimilation systems have been found in APEC, and several have had their roles in APEC pathogenesis confirmed in animal models of disease (27, 36, 48). However, the uniqueness of serum as a niche for APEC growth likely extends far beyond iron limitation. In an effort to increase our understanding of APEC's ability to cause septicemia, we performed a genome-wide transcriptional analysis of APEC O1 during its growth in chicken serum.

MATERIALS AND METHODS

Bacterial strains, plasmids, oligonucleotide primers, and growth conditions. APEC O1, a sequenced O1:K1:H7 reference strain that shares strong similarities with human extraintestinal pathogenic *E. coli* (ExPEC) genomes (27), was used in these studies. All other *E. coli* strains, plasmids, and oligonucleotide primers used in this study are listed in Table 1. Bacterial cultures were grown at 37°C in Luria-Bertani (LB) broth or chicken serum (Sigma). When necessary, antibiotics

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TABLE 1. Bacterial strains, plasmids, and primers used in this study

Strain, plasmid, or primer target	Description or sequence ^a	Reference
Strains		
APEC O1	O1:K1:H7; <i>fyuA sitA chuA irp2 iroN ireA tsh iucD fimC iss ompA vat traT</i> ; contains four plasmids, including pAPEC-O1-ColBM	27
JW1116-1	<i>E. coli</i> BW25141 derivative, $\Delta phoP790::kan$	4
JW0013-4	<i>E. coli</i> BW25141 derivative, $\Delta dnaK734::kan$	4
JW0014-1	<i>E. coli</i> BW25141 derivative, $\Delta dnaJ735::kan$	4
APEC O1 M _{dnaK}	APEC O1 derivative, $\Delta dnaK$	This study
APEC O1 M _{dnaJ}	APEC O1 derivative, $\Delta dnaJ$	This study
APEC O1 M _{phoP}	APEC O1 derivative, $\Delta phoP$	This study
APEC O1 M _{ybtA}	APEC O1 derivative, $\Delta ybtA$	This study
Plasmids		
pKD46	Apr; expresses λ red recombinase	14
pKD3	<i>cat</i> gene, template plasmid	14
Targets of primers for gene mutagenesis		
<i>phoP</i>		This study
Forward	CCAGTCAGGCTGGATCATCT	
Reverse	GAAAGAGCTGACTCGCGGTA	
<i>dnaK</i>		This study
Forward	CTTGGCTGCGATTCACTTCTT	
Reverse	AATATCGCCGAAAACGTCAC	
<i>dnaJ</i>		This study
Forward	GGTCTGAACGAAGATGAAATCC	
Reverse	TAGAGAAAAGCCCCGAGTGATA	
<i>ybtA</i>		This study
Forward	<u>ATACCCGCATTGGTCTAAGCCACAGGGAGATAACCAGGTCATGGTGTAGGC</u> TGGAGCTGCTTCGA	
Reverse	<u>GGCCTCTGTACAGGGAGGAGTTTAGGGGGCGCGACCCGGCATATGAATA</u> TCCTCCTTAG	
Targets of primers for real-time PCR		
<i>chuA</i>		34
Forward	CAGATAACAAAAGGAGCAAGGC	
Reverse	CTGACGATAATACTCACC GCC	
<i>sitA</i>		34
Forward	TGTCGCCAGATAATGCTCTG	
Reverse	TAAGTATCGGCATTGCGTTG	
<i>ybtA</i>		This study
Forward	CCGATTCGAGAGCATTACCC	
Reverse	CAACAGCAGTGGGAGTCGAT	
<i>fyuA</i>		This study
Forward	CAAACCTCCCCAGAGTCTTGC	
Reverse	CATCGACATACAGGGTGACG	
APEC O1_2945		This study
Forward	AGCCATGTCTCGTTACAGGAAT	
Reverse	GGAGAGGGCAGATTTTCACTA	
APEC O1_2950		This study
Forward	CAGGGATACGATGTTTTTCACC	
Reverse	TGCAACATTACGTCGATCTCTT	
<i>dnaK</i>		This study
Forward	GAAGCTAACGCCGAAGCTGA	
Reverse	TCAACCTGCTTACGGGTGCT	

Continued on following page

TABLE 1—Continued

Strain, plasmid, or primer target	Description or sequence ^a	Reference
<i>yfaE</i>		This study
Forward	CACAATGTGGCGGTTGAGTA	
Reverse	AGCTAACGGTTCGGCAATC	
<i>glpA</i>		This study
Forward	GGATCACCGCATTAATCAGC	
Reverse	AAAGAGGTGGTGCCAATCAG	
<i>lldR</i>		This study
Forward	GGACGTTCTGTTTTTCATCTGG	
Reverse	TGCTTCACAGAGGATTGCAG	
<i>glpF</i>		This study
Forward	AGGATCAGCCCCATCAGAAT	
Reverse	GTTGATCTGGCTGGCACTTT	
<i>tus</i>		50
Forward	CGATAACCTTTTCGCAAGCAGCGTT	
Reverse	GGCAAATGACGATGCACCCATTCA	

^a The underlined portions of the primer sequences are identical to the flanking regions of the target genes.

were added at the following concentrations: ampicillin (Ap), 50 µg ml⁻¹; kanamycin (Kn), 50 µg ml⁻¹; and chloramphenicol (Cm), 25 µg ml⁻¹. For growth kinetics, overnight cultures of *E. coli* strains were diluted 1:100 in LB medium and incubated at 37°C with shaking at 160 rpm until an optical density (OD) at 600 nm (OD₆₀₀) of 1.0 (Eppendorf biophotometer) was attained. These cultures were again diluted 1:100 into fresh LB or chicken serum to give parallel cultures representing the test conditions and controls and then incubated at 37°C until the stationary phase was reached. Growth was monitored by measuring the OD₆₀₀ and confirmed by obtaining viable counts.

RNA isolation, cDNA synthesis, labeling, microarray, and hybridization. RNA from APEC O1, cultured in both LB and chicken serum, was extracted using an RNeasy minikit (Qiagen) with a 1-h on-column DNase digestion (Qiagen) according to the manufacturer's instructions. Four biological replicates were used for each culture condition. The concentration of RNA was determined using a spectrophotometer (ND-1000; NanoDrop). Five micrograms of total RNA was used for labeling, and cDNA was synthesized and labeled with Fairplay III microarray labeling kit (Agilent Technologies-Stratagene Products) according to the recommendations of the manufacturer and labeled with the Cy3 or Cy5 monoreactive dye pack (GE Healthcare). Labeled cDNA was purified and quantified according to the manufacturer's instructions. The 8 × 15,000 (15K) DNA high-density microarray of APEC O1 was designed by Oxford Gene Technology (Oxford OX5 1PF, United Kingdom) and validated by the University of Birmingham *E. coli* Centre (UBEC) (United Kingdom). During validation, three 60-mer probes per predicted gene were designed for the 5,042 open reading frames (ORFs) in the chromosome and 4 plasmids of APEC O1. For each of the designed probes, a mismatch probe (containing 3 mismatches per 60-mer probe at positions 10, 25, and 40) was also generated. These mismatch probes and the perfect-match probes were placed on an array (4 × 44k) in triplicate. This array was hybridized with genomic DNA and a pool of mRNA representing conditions in which as many genes as practicable would be induced (derived from an equimolar pool of total RNA from *E. coli* grown in morpholinepropanesulfonic acid (MOPS) minimal medium at 30°C mid-log phase, 37°C for mid-log phase, and 37°C for stationary phase). The results were processed to select the best-performing probe for each gene. This derived and optimized probe set of 5,063 probes was printed in triplicate by Agilent Technologies on the 8 × 15K array used in this study. The probes are also arranged randomly by design. For each of four biological replicates, equal quantities of Cy5- and Cy3-labeled cDNA from APEC O1, cultured in chicken serum or LB, were added to hybridization solution, and hybridization was performed using the Gene Expression hybridization kit (Agilent Technologies).

Data and statistical analysis. Slides were scanned with a GenePix 4000B scanner (Axon Instruments), and the fluorescence intensities were collected with Image software (BioDiscovery, El Segundo, CA). Median signal intensities for each spot were background corrected and log transformed. The LOWESS procedure was used to correct the intensity-dependent dye bias for each 2-color array (15). Linear models (51) were then fitted to the normalized data for each

gene. The moderated *t* test was applied using the R software package program limma to detect the genes differentially expressed between APEC O1 grown in chicken serum and that grown in LB (51). The set of *P* values was converted to *q* values for false discovery rate control using the R package program *q* value developed by Storey et al. (54). Along with *q* values, estimates of fold change were computed.

Real-time qPCR. Real-time quantitative reverse transcription-PCR (RT-PCR) (qPCR) was used to validate the expression levels for selected genes. Total RNA from APEC O1, cultured in LB and serum, was extracted as described above. Treatment of total RNA with DNase was performed, followed by subjecting to purification using the RNeasy kit. RNA samples without reverse transcription served as PCR templates to confirm that they were free of contaminating DNA. One microgram of total RNA was reverse transcribed in triplicate using random hexamers and Moloney murine leukemia virus (MMLV) reverse transcriptase (Promega). Primer pairs used are listed in Table 1, and real-time PCR was performed as previously described (34). Melting-curve analyses were performed after each reaction to ensure amplification specificity. Differences (*n*-fold) in transcripts were calculated using the relative comparison method, and amplification efficiencies of each primer set were verified as described by Schmittgen et al. (48a). RNA levels were normalized using the housekeeping gene *tus* as a control (50).

Mutant generation and *in vitro* and *in vivo* virulence assays. Deletion of selected genes was achieved using the method of Datsenko and Wanner (14). The Kn resistance cassette, flanked by 5' and 3' sequences of the *phoP*, *dnaK*, or *dnaJ* gene, was amplified from genomic DNA of the mutant strains of *E. coli* BW25141 (Table 1) (4), while the Cm resistance cassette, flanked by 5' and 3' sequences of *ybtA*, was amplified from plasmid pKD3 (14). The primers used for mutagenesis are listed in Table 1. The Kn or Cm cassette was introduced into APEC O1 by homologous recombination using λ Red recombinase. For both *in vitro* and *in vivo* competition assays, cultures of the mutant and wild-type strains were mixed at a ratio of 1:1. For *in vitro* competition, the bacteria were incubated in LB broth for 4 h at 37°C and then plated on medium with or without antibiotics (Kn or Cm). For *in vivo* competition, 10 1-day-old chickens were infected with approximately 10⁷ CFU of the mixed culture by the intratracheal route. At 24 h postinfection, birds were euthanatized by CO₂ inhalation, lungs were collected, weighed, and homogenized, and dilutions of the homogenates were plated on medium with or without antibiotics for selection of mutants or total bacteria, respectively. A competitive index (CI) was calculated for each mutant by dividing the output ratio (mutant/wild type) by the input ratio (mutant/wild type). Values greater than 1 indicated that the mutant outcompeted the wild type, whereas values less than 1 indicated that the wild type outcompeted the mutant.

Microarray data accession number. Microarray data are available at the National Center for Biotechnology Information Gene Expression Omnibus database (<http://www.ncbi.nlm.nih.gov/geo>) under accession number GSE25478.

FIG. 1. Growth of APEC O1 in LB and chicken serum. Optical density (OD₆₀₀) (A) or CFU counts (B) were used to assess the growth of APEC O1 in culture medium LB (□) and chicken serum (■). APEC O1 grew faster in serum than in LB, suggesting that the APEC O1 can rapidly adapt to chicken serum.

RESULTS

APEC O1 rapidly adapts to chicken serum. In an effort to understand the mechanisms that enable APEC to survive in the bloodstream, APEC O1 was grown in 100% chicken serum. Initial experiments were performed to assess the growth of APEC O1 in chicken serum and LB by measuring the optical density (OD₆₀₀). At the beginning, the OD values of APEC O1 grown in serum were only slightly lower than those of APEC O1 grown in LB. However, after 5 h of incubation, the OD values differed to some extent, reaching a maximum level of difference (OD in LB, 5.39; OD in serum, 3.13) after 8 h of incubation (Fig. 1a). The growth of APEC O1 in serum and that in LB were also compared using viable counts (Fig. 1b). After 2 h of incubation, APEC O1 grown in serum lagged behind APEC grown in LB (CFU in LB, 4.1×10^7 /ml; CFU in serum, 2.2×10^7 /ml). Four hours after inoculation, viable counts of APEC in serum were higher than those in LB (CFU in LB, 4.3×10^8 /ml; CFU in serum, 6.2×10^8 /ml), with differences of nearly 2-fold reached at 6 and 8 h of incubation, although their OD values were lower. These results suggested that APEC O1 rapidly adapts to serum.

Transcriptome alteration of APEC O1. To further understand the mechanisms by which APEC adapts to serum, we used a DNA microarray to compare the transcriptome of APEC O1 following growth in LB and chicken serum. Care was used in handling the serum so as not to inactivate its complement components to ensure that our analysis would identify genes which enable complement resistance, a trait that shows strong correlation with APEC virulence (61). The expression level of each ORF in the APEC O1 genome was determined in serum relative to that for growth in LB. Genes

with at least a 1.5-fold change and a q value of <0.10 (the false discovery rate was controlled at 10%) were considered significantly differentially expressed. Overall, 311 genes were found to be significantly upregulated, while 299 genes were significantly downregulated. The heat map in Fig. 2 presents an overview of the differentially regulated genes within each functional category in the serum and LB. Growth in serum caused upregulation of most genes involved in biosynthesis of cofactors, prosthetic groups, and carriers while also leading to downregulation of genes involved in energy metabolism (Fig. 2). Thirty-nine of the upregulated genes were localized to genomic islands of APEC O1 that are not present in the *E. coli* K-12 genome (Table 2) (27). In addition, many genes contained within three plasmids of APEC O1, one of which is considered to be cryptic (17), were significantly upregulated under these same conditions (Table 3). Fifty-six genes found within APEC O1's large virulence plasmid, pAPEC-O1-ColBM, were significantly upregulated in chicken serum (Table 3), suggesting that this virulence plasmid is important in APEC O1's adaptation to chicken serum.

To confirm the results from the microarray experiments, real-time quantitative reverse transcription-PCR was performed on the 11 genes listed in Table 1, in which the expression of 7 of these genes was upregulated and that of the other 4 was downregulated, as evidenced by microarray results. The qPCR results were consistent with the results obtained from the microarray experiments (Fig. 3). However, for most of these genes, the qPCR results showed fold changes greater than those seen with the corresponding microarray results. Therefore, results of the qPCR analysis provided evidence that the transcriptional data obtained from microarray experiments were reliable, indicating that the rejection level applied to the microarray data was adequate.

Virulence traits. Several traits are known to contribute to APEC virulence. The expression of several of these "known" loci was affected by exposure to chicken serum. We found that the genes involved in iron acquisition were among the genes most significantly upregulated. Among the top 20 most significantly upregulated genes, 13 were involved in iron uptake. The yersiniabactin system (46) was the most significantly upregulated iron acquisition system in APEC O1, with the *ybtA* gene upregulated more than 11-fold in serum compared to results in LB. Other significantly upregulated systems included the genes of the aerobactin (56), enterobactin (60), and *sit* operons (63), plus the *chu* genes, which are required for hemin utilization (25). Also, the expression of some genes involved in ferrisidrophore complexes, such as *fluA* and *fep*, was also significantly upregulated.

Stress response to serum. In addition to the failure to detect upregulation of "known" complement resistance mechanisms, one of the most interesting findings in this study was that several categories of genes involved in stress resistance were upregulated during incubation in serum. The genes *uspG* and *uspA*, which contribute to universal stress resistance (32), were significantly upregulated when APEC O1 was grown in serum. Exposure to serum also resulted in upregulation of such acid resistance genes as *hdeA*, *hdeB*, *gadA*, *gadB*, and *gadX* (19), although the pH value of the serum used in this study was 8.20.

Several genes associated with oxidative stress in APEC O1 were upregulated during serum growth. *sodA*, which encodes

FIG. 2. Heat map visualizing regulated genes in APEC O1 grown in chicken serum compared to growth in LB. Genes found to be significantly regulated are indicated by either green (upregulation) or red (downregulation). Significantly differentially expressed genes of APEC O1 during growth in serum compared to that in LB are listed in columns 2 and 3. The genes are sorted by functional categories (column 1) according to annotation by the J. Craig Venter Institute.

an *E. coli* superoxide dismutase, was upregulated more than 1.5-fold after exposure to serum (44). Also, the gene *katE*, a well-known gene that participates in the antioxidant defense mechanism against H_2O_2 -induced stress in *E. coli* (44), was significantly upregulated in serum. In addition to genes participating in H_2O_2 resistance, *msrA* and *dps* were identified as being significantly upregulated. They are predicted to protect cells and DNA from oxidative damage (49).

Also, several genes contributing to heat shock protection were significantly upregulated, though APEC O1 was cultured in serum at 37°C. The genes of the *rpoH* regulon, including *dnaK*, *dnaJ*, *hchA*, *ibpA*, *clpB*, and *hspG* (20), were upregulated when APEC O1 was cultured in serum at 37°C. To determine if heat shock genes contribute to APEC virulence, we deleted *dnaK* and *dnaJ* from APEC O1 and performed *in vitro* and *in vivo* competition assays using the mutants and the wild type. Since *rpoH* is an essential gene for *E. coli*, it was not possible to test its contribution to virulence by deletion analysis. The wild type significantly outcompeted the two heat shock mutants for growth in day-old chickens (Table 4), despite the fact that the mutants grew only slightly slower than the wild type *in vitro* at 37°C, confirming that the heat shock genes of the *rpoH* regulon are involved in APEC virulence.

Adaptive metabolic shift during growth in chicken serum. The growth of APEC O1 in serum resulted in a substantial transcriptional response in genes involved in metabolism, reflecting a need for rapid adaptation to the nutritional conditions encountered by APEC in serum. Among these changes was an alteration in APEC O1's preference for nutrient utilization. Increased expression of a gluconate binding protein

(YhgH) and APEC O1_3052, which encodes a gluconate transport-associated protein, was also observed. Significant inhibition was observed in glycerol metabolic genes, which were downregulated by 10- to 25-fold. Also, cultivation in serum resulted in inhibition of genes involved in catabolism of amino sugars, *N*-acetylglucosamine (NAG) and *N*-acetylneuraminic (sialic) acid; C4-dicarboxylates, fumarate and succinate; and the amino acids, serine, tryptophan, and aspartate.

Several genes involved in central metabolism were significantly upregulated after APEC O1 was transferred from LB to serum. There was an increase in the expression of three central metabolic enzymes that are involved in the pentose phosphate pathway (PP pathway). The gene *gcd*, which encodes a glucose dehydrogenase and is used in the oxidative PP pathway, was upregulated 2.18-fold. In addition, two genes, *talA* and *tktB*, encoding two nonoxidative pentose phosphate pathway enzymes (53), were upregulated 2.16- and 2.19-fold, respectively. The transaldolase (TalA) converts sedoheptulose-7-phosphate and glyceraldehyde-3-phosphate to erythrose-4-phosphate and fructose-6-phosphate, while the transketolase catalyzes the reverse reaction, the conversion of sedoheptulose-7-P and glyceraldehyde-3-P to the aldose D-ribose-5-P and ketose D-xylulose-5-P.

Growth in serum influences transport. In addition to differential regulation of iron transport systems (described in the virulence section), exposure of APEC O1 to serum also changed the transcription of a number of genes encoding other transport systems. The manganese transport protein MntH (47) was significantly upregulated more than 3-fold, and the copper transporter YbaR was upregulated 2.8-fold. These re-

TABLE 2. Significantly upregulated genes of APEC O1 localized in genomic islands that are not present in *E. coli* K-12

Gene locus	Gene name	Function of product	Genomic island no. ^a	Fold change in expression	P value	q value
APECO1_274	<i>sitA</i>	ABC Mn ²⁺ /Fe ²⁺ transporter	9	10.8375	3.49E-07	4.40E-05
APECO1_273	<i>sitB</i>	ABC Mn ²⁺ /Fe ²⁺ transporter	9	8.816332	6.27E-08	2.99E-05
APECO1_272	<i>sitC</i>	ABC Mn ²⁺ /Fe ²⁺ transporter	9	8.502317	9.67E-07	6.60E-05
APECO1_271	<i>sitD</i>	ABC Mn ²⁺ /Fe ²⁺ transporter	9	7.292451	1.90E-06	0.000101
APECO1_246		Hypothetical protein	9	3.891812	2.06E-05	0.000508
APECO1_275		Hypothetical protein	10	1.671198	0.000918	0.006928
APECO1_277	<i>ycgE</i>	Transcriptional regulator	10	1.606007	0.005808	0.021801
APECO1_391		Hypothetical protein	11	1.702988	0.000305	0.003246
APECO1_1020	<i>gp7</i>	Hypothetical protein	15	1.61494	0.011325	0.034143
APECO1_1036		Hypothetical protein	15	1.546323	0.005074	0.019965
APECO1_1057		Putative AraC type regulator	16	11.80515	1.25E-06	7.55E-05
APECO1_1063	<i>fyuA</i>	Yersiniabactin receptor protein	16	2.80218	5.62E-06	0.000215
APECO1_1067		Transporter	16	2.306758	1.58E-05	0.000429
APECO1_1092		Hypothetical protein	16	2.086762	4.67E-05	0.000899
APECO1_1058	<i>irp2</i>	Yersiniabactin biosynthetic protein	16	1.997221	0.000811	0.006389
APECO1_1059	<i>irp1</i>	Yersiniabactin biosynthetic protein	16	1.959211	9.01E-05	0.001376
APECO1_1052		Salicylate synthase Irp9	16	1.663941	0.000526	0.004743
APECO1_1105	<i>yeeX</i>	COG2926S;hypothetical protein	16	1.659641	0.003874	0.016885
APECO1_39122		Hypothetical protein	22	2.031662	0.000108	0.001554
APECO1_3897		Hypothetical protein	22	1.72608	0.00025	0.002821
APECO1_3497	<i>ireA</i>	Iron-regulated element	25	3.5312	1.35E-05	0.000379
APECO1_3530	<i>tia</i>	Tia invasion determinant	25	1.734973	0.000748	0.006046
APECO1_3385	<i>fepC</i>	Enterobactin transport	28	3.664637	2.16E-05	0.000518
APECO1_3389		Putative phosphosugar isomerases	28	2.734085	6.79E-06	0.000246
APECO1_3386		Protein of ABC transporter family	28	2.188546	0.000239	0.002726
APECO1_3388		Protein of ABC transporter family	28	2.076194	6.10E-05	0.00105
APECO1_2947	<i>chuT</i>	Outer membrane binding protein	32	10.76088	2.51E-07	4.36E-05
APECO1_2948	<i>chuA</i>	Heme/hemoglobin receptor	32	10.12316	7.87E-08	3.05E-05
APECO1_2946	<i>chuW</i>	n III oxidase	32	9.730668	2.17E-06	0.000107
APECO1_2945		Hypothetical protein	32	8.480435	4.11E-07	4.40E-05
APECO1_2944		Hypothetical protein	32	7.263724	3.14E-07	4.40E-05
APECO1_2949	<i>chuS</i>	Heme/hemoglobin transport	32	5.863794	2.89E-07	4.36E-05
APECO1_2950	<i>yhiF</i>	Transcriptional regulator	32	3.361944	1.28E-05	0.000372
APECO1_2306		Insertion element	40	4.556443	5.59E-07	5.07E-05
APECO1_2257	<i>insB</i>	COG1662L;IS1 InsB protein	40	1.896451	0.000155	0.001939
APECO1_2262	<i>insA</i>	COG3677L;IS1 InsA protein	40	1.803386	0.000129	0.001727
APECO1_2256	<i>insA</i>	COG3677L;IS1 InsA protein	40	1.752979	0.000752	0.006046
APECO1_2279		Putative hemolysin activator protein	40	1.606824	0.014772	0.040738
APECO1_2024		Hypothetical protein	43	1.636221	0.002654	0.013566

^a Genomic island no. according to Johnson et al., 2007 (27).

sults suggest that in addition to being a low-iron environment, serum is a low-manganese and -copper environment and metal ion limitation presents a significant obstacle to APEC infection. Interestingly, eight genes encoding a type II secretion system (T2SS), including *gspC*, *gspD*, *gspE*, *gspF*, *gspH*, *gspI*, *gspK*, and *gspL*, were significantly downregulated when APEC O1 was cultured in serum compared to results in LB (12).

Regulators. Several regulatory genes, including regulators of unknown function, showed altered expression in serum. We found that a putative regulator encoded by the *Yersinia* high-pathogenicity island (HPI) (27) was significantly upregulated in serum. This gene (APECO1_1057) is localized immediately upstream of the gene *irp2* of the HPI and is identical to the gene *ybtA* of HPI. A mutant generated by nonpolar deletion of this gene was shown to lead to APEC attenuation using the *in vivo* competition assay (Table 4).

PhoP/PhoQ is a known two-component signal transduction system. It responds to antimicrobial peptides and a low Mg²⁺ concentration and is involved in bacterial virulence. Several genes belonging to the *phoP* regulon, including *yrbL*, *slyB*, *gadA*, *hdeA*, *hdeB*, *gadX*, and *ompT* (39), were significantly

upregulated in serum. However, its importance in extraintestinal pathogenic *E. coli* (ExPEC) virulence has never been demonstrated. We deleted *phoP* from APEC O1 and tested the mutant in the *in vivo* competition assay (Table 4). The *phoP* mutant was attenuated compared to the wild type, further suggesting that PhoP plays a role in APEC virulence.

Expression of two two-component signal transduction systems (TCSs) was reduced in the microarray analysis. The sensor histidine kinase gene of TCS, encoding PmrA/PmrB, was found to be significantly downregulated. TCS PmrA/PmrB mediates *Salmonella* resistance to stress of Fe(III), Al(III), and antimicrobial peptides by modifying LPS expression (41). The response regulator NarP of NarQ/NarP was also downregulated. This TCS activates gene expression in response to nitrate and is especially sensitive to low concentrations of nitrate (58).

DISCUSSION

The ability to survive and grow in chicken serum appears to be an important virulence determinant for APEC strains and may play a major role in the pathogenesis of avian colibacillosis

TABLE 3. Significantly upregulated genes from plasmids of APEC O1 cultured in chicken serum compared to results with LB

Gene/locus name	Function or description of product	Fold change in expression	P value	q value
pAPEC-O1-ColBM_143	Putative integrase	36.16894	6.60E-08	2.99E-05
pAPEC-O1-ColBM_142	Hypothetical protein	23.16964	5.49E-08	2.99E-05
pAPEC-O1-ColBM_138	Salmochelin system component	14.35178	8.22E-07	5.93E-05
pAPEC-O1-ColBM_135	Hypothetical protein	14.04616	9.74E-07	6.60E-05
<i>insA</i>	Hypothetical protein	11.40321	2.23E-07	4.36E-05
<i>iroB</i>	Salmochelin system component	9.474809	1.56E-06	8.50E-05
<i>sitA</i>	ABC Mn ²⁺ /Fe ²⁺ transporter	8.833535	2.68E-07	4.36E-05
<i>iucC</i>	Aerobactin siderophore system	8.818279	4.08E-07	4.40E-05
<i>sitC</i>	ABC Mn ²⁺ /Fe ²⁺ transporter	8.212306	3.61E-07	4.40E-05
<i>sitB</i>	ABC Mn ²⁺ /Fe ²⁺ transporter	7.501994	9.74E-08	3.31E-05
pAPEC-O1-ColBM_136	Hypothetical protein	7.247297	2.00E-06	0.000103
<i>crcB</i>	Hypothetical protein	6.613707	4.21E-07	4.40E-05
<i>iucB</i>	Aerobactin siderophore system	6.417662	6.95E-07	5.72E-05
<i>iucA</i>	Aerobactin siderophore system	6.05921	1.13E-06	7.29E-05
<i>iroC</i>	Salmochelin system component	5.455652	3.12E-06	0.000143
<i>iucD</i>	Aerobactin siderophore system	5.194098	1.44E-06	8.12E-05
pAPEC-O1-ColBM_126	IS2 transposase subunit	5.168253	5.12E-07	4.97E-05
pAPEC-O1-ColBM_149	S2 transposase	5.075606	5.02E-07	4.97E-05
<i>shiF</i>	Putative transport protein	5.048586	1.10E-05	0.000347
pAPEC-O1-ColBM_94	Hypothetical protein	4.373617	9.96E-07	6.60E-05
pAPEC-O1-ColBM_150	Hypothetical protein	4.110728	7.65E-06	0.000263
pAPEC-O1-ColBM_125	Transposase for IS2	4.094815	4.45E-06	0.000189
<i>iroD</i>	Salmochelin system component	3.924875	8.79E-06	0.000295
<i>iroN</i>	Salmochelin system component	3.179762	0.000814	0.006392
pAPEC-O1-ColBM_132	Hypothetical protein	3.123875	2.44E-05	0.000565
pAPEC-O1-ColBM_95	IS2 transposase	2.866879	0.000107	0.001554
<i>cvaA</i>	Hypothetical protein	2.553727	1.11E-05	0.000347
pAPEC-O1-ColBM_1	IS1 <i>InsA</i> protein	2.526474	8.48E-06	0.000288
<i>eitC</i>	Putative iron transport system	2.42768	2.12E-05	0.000517
pAPEC-O1-ColBM_74	Transposase for IS1	2.024255	0.000388	0.003795
<i>sitD</i>	ABC iron transport system	1.882365	0.00037	0.003676
pAPEC-O1-ColBM_133	Hypothetical protein	1.870467	0.001961	0.011098
<i>cvaB</i>	Assembly verified for accuracy	1.860303	0.004612	0.018642
<i>eitB</i>	Putative iron transport system	1.8456	0.000255	0.002854
<i>relE</i>	Cytotoxic translational repressor	1.805647	0.002744	0.01381
pAPEC-O1-ColBM_69	Hypothetical protein	1.799338	0.003073	0.014697
<i>etsA</i>	Putative transport system	1.779548	0.000281	0.003057
<i>eitD</i>	Putative iron transport system	1.772026	0.000131	0.00173
<i>insB</i>	Hypothetical protein	1.767588	0.000153	0.001919
<i>insL</i>	Transposase	1.757236	0.001065	0.007573
<i>sopA</i>	Plasmid partitioning protein	1.745449	0.000263	0.002926
<i>traY</i>	Conjugal transfer protein	1.729107	0.00036	0.003622
pAPEC-O1-ColBM_167	Hypothetical protein	1.727454	0.000894	0.006819
<i>eitA</i>	Putative iron transport system	1.718027	0.003175	0.015078
<i>iutA</i>	Aerobactin siderophore system	1.671313	0.001793	0.010636
<i>insB</i>	Hypothetical protein	1.66898	0.000938	0.006979
pAPEC-O1-ColBM_160	Hypothetical protein	1.666459	0.000377	0.003727
<i>etsB</i>	Putative transport system	1.659028	0.000583	0.005023
<i>relE</i>	Cytotoxin translational repressor	1.647127	0.009351	0.029787
pAPEC-O1-ColBM_185	Hypothetical protein	1.639247	0.005399	0.020854
<i>eno</i>	Putative enolase	1.608608	0.001787	0.010636
<i>etsC</i>	Putative transport system	1.599998	0.001159	0.00799
pAPEC-O1-ColBM_187	Hypothetical protein	1.585905	0.00394	0.017012
pAPEC-O1-ColBM_175	Hypothetical protein	1.582197	0.000443	0.00416
<i>traQ</i>	Pilin chaperone protein	1.53625	0.016134	0.043008
pAPEC-O1-ColBM_4	Hypothetical protein	1.500467	0.000909	0.00692
pAPEC-O1-R_91	Hypothetical protein	1.742547	0.004732	0.01896
pAPEC-O1-Cryp2_19	Hypothetical protein	1.649976	0.000361	0.000362
pAPEC-O1-R_194	Hypothetical protein	1.526988	0.001155	0.033743

(37). Here, we describe the first pan-genome microarray analysis of the global transcriptional response of APEC to chicken serum using the first APEC genome-specific microarray. Previous studies of APEC using transcriptional analysis compared expression of 152 virulence genes of APEC *in vivo* (62) and

sought to identify the consequences of a *pst* mutation (13). Our study provides insight into the global gene regulation and the interplay required for adaptation to serum, a condition for growth that was used to mimic the conditions that APEC encounters during host infection.

FIG. 3. Comparison of gene regulation analyzed by microarray (filled) or real-time quantitative RT-PCR (open). Real-time quantitative RT-PCR was used to validate the expression level for 11 selected genes, including 6 upregulated genes and 4 downregulated genes revealed by microarray analysis.

The microarray results presented here are internally consistent and often are compatible with those in previous studies. All iron uptake systems were significantly upregulated, which is consistent with the knowledge that serum is a low-iron environment (10). Also, genes within the same iron uptake operons (i.e., the enterobactin, *sit*, and *chu* operons) were uniformly upregulated, suggesting that there is coordinated regulation of iron acquisition genes within an operon. Several iron uptake systems, such as *sit*, *chu*, aerobactin, and *iro*, have been associated with APEC virulence in previous studies (8, 36, 48, 52), and our results confirm their importance in APEC pathogenesis. Also, the contribution of APEC O1's large pathogenicity island (PAI)-containing plasmid, pAPEC-O1-ColBM, to virulence was demonstrated here. Fifty-six genes that are mostly localized to the conserved regions of APEC's plasmid PAIs (26, 28) were significantly upregulated. While the biological functions of some of these upregulated plasmid genes are known and include iron uptake, adhesion, plasmid replication, and stability, many were of unknown function. This strong representation of "genes of unknown function" among those upregulated under host-like conditions underscores how much there is to learn about *E. coli*'s complement resistance and virulence and how little we know about the contributions of plasmids to the pathogenesis of disease. Further studies to delineate the functions of these genes certainly seem justified.

Since colonization is a first step in bacterial infections, it is notable that none of APEC O1's known fimbrial gene clusters were upregulated. That is, of the 9 fimbrial gene clusters pre-

dicted to occur in APEC O1 (3, 27), all were either not significantly changed or mostly downregulated. In addition, the expression of the autotransporter adhesin AatA, which was previously described as being upregulated in serum (35), did not significantly change. However, the nonfimbrial adhesin gene *tia*, which was previously localized to PAI I_{APEC-O1} in APEC O1, was upregulated significantly (30). This gene is identical to ETEC *tia* and shares significant homology with *hraI* from porcine enterotoxigenic *E. coli* (ETEC) and limited homology with the *ail* locus from *Yersinia* spp. (18). The encoded 25-kDa Tia OMP plays a role in ETEC's adherence of epithelial cells; however, the role of *tia* in APEC pathogenesis has yet to be determined. A recent study suggested that APEC contains adhesins that have not yet been identified (2), and Tia may be one such candidate.

In addition to these findings, our microarray data present provocative contrasts to previous reports on the mechanisms involved in *E. coli*'s complement resistance (11, 40, 42, 59). A number of bacterial cell surface structures, including capsule, LPS, and OMPs, have been previously associated with resistance of *E. coli* to serum. In contrast, neither genes involved with K1 capsular synthesis nor LPS synthesis genes were upregulated in APEC O1 when it was grown in serum. Similarly, three OMP genes, *ompA*, *iss*, and *traT*, thought to contribute to *E. coli*'s serum resistance (11, 40, 42, 59), were not upregulated in APEC O1 when grown in serum. At this time, it is not known whether the conditions used (incubation at 37°C and pH 8.2) are responsible for this observation. If not, this may suggest that some virulence genes may be constitutively expressed *in vivo* and *in vitro*.

One of the most provocative findings in this study is the identification of the role that adaptation to stress plays in serum resistance. Several genes involved in resistance to general, acid, oxidative, and heat stress were upregulated in serum, confirming that serum is a stressful environment for APEC. During growth in serum, expression of the superoxidase protein, SodA, and the H₂O₂ scavenger protein, KatE, was upregulated. This suggests that APEC differentially regulates expression of these systems to cope with oxidative stress encountered during growth in serum. Superoxide (O₂⁻) is generated when *E. coli* produces ATP via the respiratory chain, and SodA, which exists in the bacterial cytoplasm, can convert O₂⁻ to H₂O₂. APEC cells may also be exposed to H₂O₂ produced by phagocytes during infection. Since H₂O₂ can produce cell damage, it must be removed or detoxified. Thus, such mechanisms, which confer the ability to resist oxidative stress, are associated with bacterial virulence (21, 23).

TABLE 4. *In vitro* and *in vivo* competition index of selected mutants

Gene	Function of product	Fold change in expression	<i>P</i> value	<i>q</i> value	CI ^{a,b}	
					<i>In vitro</i>	<i>In vivo</i>
<i>dnaK</i>	Chaperone protein	7.17442	0.001626	0.033028	0.88 (0.23–0.46)	0.43 (0.08–0.11)
<i>dnaJ</i>	Chaperone protein	4.08406	0.001597	0.032868	0.91 (0.19–0.33)	0.50 (0.13–0.25)
<i>phoP</i>	Response regulator in TCS ^c	3.690768	0.009174	0.065915	0.76 (0.29–0.46)	0.23 (0–0.35)
<i>ybtA</i>	Regulator	7.828241	1.06E-05	0.00089	1.03 (0.24–0.68)	0.35 (0–0.28)

^a CI, competitive index. Values > 1 indicate that the mutant outcompeted the wild type; values < 1 indicate that the wild type outcompeted the mutant.

^b Values are the mean CI values obtained from 5 to 10 chicks each, along with upper and lower confidence limits. Values in bold are significant from 1 (*P* < 0.05).

^c TCS, two-component signal transduction system.

Besides the upregulation of oxidative resistance genes, five heat shock genes, all belonging to the *rpoH* regulon, were significantly upregulated when APEC O1 was grown in serum at 37°C. The product of *rpoH*, σ^{32} , is the major transcriptional activator of the heat shock genes. However, the bacterial heat shock response is not limited to changes in temperature alone and is regarded as a general stress response (20). Our data showing that deletion of several heat shock proteins led to attenuation of APEC O1 in chickens confirm that these genes contribute to APEC virulence. Thus, upregulation of the *rpoH* regulon may be necessary for APEC to resist general stress during infection or to adapt to the chicken's body temperature of 42°C. Many previous studies have demonstrated a potential correlation between pathogenesis and the heat shock response (20). However, in most cases the genes encoding these proteins are not part of the heat shock regulon, and the induction of these genes at increased temperature is mediated by other processes or is regulated only indirectly by the heat shock response (20). Here we have provided direct evidence that heat shock proteins contribute to APEC virulence.

We also found that some genes encoding metabolic enzymes are differentially expressed, which signifies metabolic adaptation for APEC grown in chicken serum. Our results demonstrated that gluconate is a preferred sugar and PP pathway may be an important route of carbon flux during APEC O1's growth in serum. Similar findings were reported for uropathogenic *E. coli* (UPEC) growing in human urine (1) and *E. coli* K-12 colonizing the mouse intestine (9). Notably, several genes involved in glycerol, amino sugars, or C4-dicarboxylate catabolism were significantly downregulated, while the corresponding genes were upregulated in UPEC (1) and *E. coli* K-12 (9).

In Gram-negative bacteria, T2SS is one of five protein secretion systems that export bacterial proteins from within the cell to the periplasmic space or the extracellular milieu or into host cells. A complete set of T2SS genes includes *gspCDEFGHIJKLMO*, are common among the proteobacteria. Type II secretion has been associated with virulence in many bacterial pathogens (12). The downregulation of T2SS in serum suggests this system may play a limited role in APEC virulence or that its importance is restricted to some particular site of infection or pathogenic process, which occurs somewhere other than in serum.

Iron is necessary for various cellular functions (57). Thus, the fact that the concentration of free iron is exceedingly low in host tissues necessitates that APEC acquire iron inside the host (57). However, excess iron is also toxic to bacterial cells. Consequently, acquisition of iron by bacteria is under the tight control of the master negative regulator Fur (57). The elegant regulation of iron acquisition in host niches also requires three other types of activators, including TCSs, extracytoplasmic function (ECF) sigma factors, and AraC-like regulators of the synthesis of siderophores and their uptake systems (22, 57). Our microarray results revealed that an AraC-like regulator, YbtA, encoded by APEC O1's HPI, was significantly upregulated during growth in serum. In *Yersinia*, this regulator induces the yersiniabactin biosynthesis operon *irp21*, *ybtUTE*, the *ybtPQXS* operon, and the Fe-yersiniabactin receptor FyuA (17). The HPI has been shown to be involved in the virulence of *Yersinia pestis*, *Y. enterocolitica*, and *Y. pseudotuberculosis* (7) and is widely distributed among APEC strains (16). However,

its role in APEC pathogenesis has never been clearly demonstrated. Deletion of *ybtA* in this study led to APEC attenuation in chickens, providing evidence that this HPI contributes to APEC O1 virulence.

TCSs are widely employed by bacteria for sensing and responding to the environment (33). Over 32 TCSs have been found in nonpathogenic *E. coli* that are also found in pathogenic *E. coli* (45). Several TCSs have been associated with the virulence of pathogenic *E. coli*, and a previous study demonstrated that the TCS BarA/UvrY contributes to APEC pathogenesis (24). Our microarray data showed that several target genes of TCS PhoP/PhoQ were significantly upregulated during APEC O1's growth in serum. Furthermore, the *phoP* deletion mutant was outcompeted by the APEC wild type, suggesting that this gene contributes to APEC virulence. The PhoQ/PhoP TCS senses external Mg^{2+} and antimicrobial peptide concentrations and controls adaptation to the *in vivo* environment. Although it is a major regulator of virulence in the enteric pathogen *Salmonella enterica* serovar Typhimurium (6) and its regulon in *E. coli* K-12 was extensively studied (39), a link between this signal transduction system and the virulence of pathogenic *E. coli* has not been established. To our knowledge, this is the first report linking this important signal transduction system with ExPEC virulence.

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