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The Type VI Secretion System Plays a Role in Type 1 Fimbria Expression and Pathogenesis of an Avian Pathogenic *Escherichia coli* Strain▽†

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Avian pathogenic *Escherichia coli* (APEC) strains frequently cause extraintestinal infections and are responsible for significant economic losses in the poultry industry worldwide. APEC isolates are closely related to human extraintestinal pathogenic *E. coli* (ExPEC) strains and may also act as pathogens for humans. Known APEC virulence factors include adhesins such as type 1 fimbriae and curli, iron acquisition systems, and cytotoxins. Here we show that APEC strain SEPT362, isolated from a septicemic hen, expresses a type VI secretion system (T6SS); causes cytoskeleton rearrangements; and invades epithelial cells, replicates within macrophages, and causes lethal disease in chicks. To assess the contribution of the T6SS to SEPT362 pathogenesis, we generated two mutants, *hcp* (which encodes a protein suggested to be both secreted and a structural component of the T6SS) and *clpV* (encoding the T6SS ATPase). Both mutants showed decreased adherence and actin rearrangement on epithelial cells. However, only the *hcp* mutant presented a mild decrease in its ability to invade epithelial cells, and none of these mutants were defective for intramacrophage replication. Transcriptome studies showed that the level of expression of type 1 fimbriae was decreased in these mutants, which may account for the diminished adhesion and invasion of epithelial cells. The T6SS seems to be important for the disease process, given that both mutants were attenuated for infection in chicks. These results suggest that the T6SS influences the expression of type 1 fimbriae and contributes to APEC pathogenesis.

Extraintestinal pathogenic *Escherichia coli* (ExPEC) strains cause a wide range of diseases, including urinary tract infections, newborn meningitis, abdominal sepsis, and septicemia (59). ExPEC pathotype subgroups include avian pathogenic *E. coli* (APEC), uropathogenic *E. coli* (UPEC), neonatal meningitis *E. coli* (NMEC), and septicemic *E. coli* (61). In poultry, APEC strains are a frequent cause of extraintestinal infections leading to respiratory or systemic disease. Colisepticemia, the most severe systemic disease, is characterized by pericarditis, perihepatitis, airsacculitis, synovitis, and peritonitis and is responsible for significant economic losses in the poultry industry worldwide (34). APEC strains are closely related to human ExPEC strains, with significant similarity existing between UPEC and APEC (46, 47). Additionally, it has been suggested that some closely related clones can be involved in extraintestinal infections in humans and poultry, suggesting no host specificity for these types of isolates (27, 43, 46, 47, 58).

The pathogenicity mechanisms of APEC are varied and remain largely uncharacterized. Established APEC virulence traits include iron uptake systems, the production of cytotoxins, and adhesins (40, 41, 76). Adhesins play an important role in APEC pathogenesis, and the best-characterized adhesins include type 1, P, and curli fimbriae and temperature-sensitive hemagglutinin (Tsh). These adhesins are required for the full virulence of specific strains probably acting in different steps of the infection process (7).

Type 1 fimbriae are produced by many strains of *E. coli*, being an important virulence determinant involved in adhesion to eukaryotic cells (5, 10, 24, 28, 32, 38, 48–50, 60, 73). This fimbria in colibacillosis has been associated with mucus adherence, colonization of the trachea and the intestinal tract, and interactions with lung epithelial cells (14, 15, 33, 39). Structural and assembly components of the type 1 fimbriae are encoded by genes within the *fim* operon, which is comprised of nine genes, including *fimA* (major subunit protein); *fimF*, *fimG*, and *fimH* (minor subunits); *fimC* (chaperone); *fimD* (usher); *fimI* (fimbria-like protein); and *fimE* and *fimB*, that encode regulators that control the invertible element that can switch the orientation of the *fim* promoter to "on" (fimbria expressed) or "off" (fimbria repressed) (5, 10, 24, 28, 32, 38, 48–50, 52, 60, 73). *csg* (curlin subunit gene) is a gene cluster that encodes the curli fimbriae and is comprised of two differently transcribed operons, one that contains the *csgB*, *csgA*, and *csgC* genes (encoding structural components of the fimbriae) and a second that contains *csgD* (encoding a transcriptional activator), *csgE*, and *csgG* (encoding an outer membrane lipoprotein involved in the extracellular stabilization of *csgA* and *csgB*) (3, 9, 25, 51). For APEC it has been shown that curli fibers are involved in

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the internalization of bacteria by eukaryotic cells, leading to avian septicemia (23). There are 11 genes encoding the P pilus organized in the *pap* gene cluster, with *papF* and *papG* encoding proteins essential for adhesion and the product of the *papC* being required for biogenesis (31).

Specialized secretion systems, which are widespread in Gram-negative bacteria, are important virulence determinants responsible for survival and replication within the host (20). Recently, a new secretion system, named the type VI secretion system (T6SS), was described for *Vibrio cholerae* (57), *Pseudomonas aeruginosa* (44), and several other bacteria (19, 20), including APEC (70), representing a new paradigm for protein secretion (19, 20). Although the exact functions of most of the proteins of the T6SS are not known, the majority of them are not secreted but are necessary for the secretion of two proteins in this system: the hemolysin-coregulated protein (Hcp) and the valine-glycine repeat protein (VgrG). Some VgrG proteins present a C-terminal extension, including effector domains that are involved in a variety of functions, such as the cross-linking of host actin, degradation of the peptidoglycan layer, and ADP-ribosylation of host proteins (35, 37, 44, 56, 57). Hcp is a protein suggested to be both secreted and structural. The crystal structure of Hcp reveals the formation of hexameric rings (44), which polymerize in solution to form tubes with an internal 40-Å diameter, approximately 100 nm long (6). It was suggested previously that Hcp tubes form the conduit through which proteins are transported to the extracellular environment or into target cells (6, 44). The T6SS protein ClpV is a member of the Hsp100/Clp ring-forming ATP that energizes the transport process. It acts by forming a hexameric channel that enables protein transport in an ATP hydrolysis-dependent manner (44, 45, 67).

Inasmuch as APEC carries a T6SS, we deleted two T6SS genes (*hcp* and *clpV*) in an APEC strain isolated from septicemic birds and demonstrated that these mutants presented diminished adhesion and invasion of *in vitro*-cultured cells, delayed and decreased pathogenicity in an *in vivo* chick infection model, and decreased expression of type 1 fimbriae. Our data suggest that T6SS genes are related to virulence and type 1 fimbria expression in APEC.

MATERIALS AND METHODS

Strains and growth conditions. Strain SEPT362 is an APEC strain (OR:H10) (streptomycin, tetracycline, and ampicillin resistant) that was isolated from the liver of a hen presenting clinical signs of septicemia. It belongs to the bacterial collection of the Bacterial Molecular Biology Laboratory of the Department of Genetics, Evolution, and Bioagents at Campinas State University (UNICAMP), Sao Paulo, Brazil. All strains and plasmids used in this study are listed in Table 1. Strains were grown aerobically in LB medium or Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) at 37°C. Antibiotics were added at the following concentrations: 100 μg ml^{-1} ampicillin, 30 μg ml^{-1} chloramphenicol, 50 μg ml^{-1} kanamycin (Km), and 25 μg ml^{-1} tetracycline. Recombinant DNA and molecular biology techniques were performed as previously described (62). All oligonucleotides used in the study are listed on Table 2.

Construction of the *hcp* and *clpV* mutants. Disruptions of the *hcp* and *clpV* genes in the chromosome of strain SEPT362 were achieved by using λ red mutagenesis (13). Briefly, primer pairs were used to amplify the Km cassette from plasmid pKD4 along with 50 nucleotides on each side of the cassette corresponding to the initial and final sequences of the gene that will promote homolog recombination (*clpV* and *hcp* genes) (Table 2). The purified PCR product was introduced by electroporation into strain SEPT362 containing λ red recombinase plasmid pKD46C (pKD46 modified by the insertion within the XmnI site of a chloramphenicol cassette amplified from plasmid pACYC184 using primers CmF and CmR), and the transformed bacterial cells were plated onto and grown on LB agar containing streptomycin and kanamycin at 37°C. In order to generate a nonpolar mutation, the Km cassette was eliminated by using plasmid pCP20. The deletion of the *hcp* and *clpV* genes in strains FDP1 (Δ*hcp*) and FDP2 (Δ*clpV*), respectively, was confirmed by PCR and DNA sequencing. The Δ*hcp* and Δ*clpV* mutants were complemented with the *hcp* and *clpV* genes cloned into the BamHI and SalI sites of plasmid pACYC184, generating plasmids pFDP3 (*hcp*) and pFDP4 (*clpV*) and generating strains FDP5 (FDP1 with pFDP3) and FDP6 (FDP2 with pFDP4).

TABLE 1. Strains and plasmids

Strain or plasmid	Relevant genotype[a]	Source or reference
Strains		
SEPT362	APEC strain isolated from a septicemic hen	This study
FDP1	*hcp* mutant	This study
FDP2	*clpV* mutant	This study
FDP5	FDP1 with pFDP3	This study
FDP6	FDP2 with pFDP4	This study
Plasmids		
pACYC184	Cloning vector	New England Biolabs
pKD4	pANTSγ derivative containing FRT-flanked kanamycin resistance	13
pKD46	λ red recombinase expression plasmid	13
pKD46C	pKD46 with a chloramphenicol cassette insertion	This study
pCP20	TS replication and thermal induction of FLP synthesis	11
pFDP3	*hcp* in pACYC184	This study
pFDP4	*clpV* in pACYC184	This study

[a] FRT, FLP recombination target; TS, temperature sensitive.

RNA extraction for microarray analyses. Cultures of wild-type (WT) strain SEPT362 and the Δ*hcp* and Δ*clpV* mutants were grown aerobically in LB medium at 37°C overnight, diluted 1:100 in DMEM (Invitrogen), and grown at 37°C and 250 rpm to an optical density at 600 nm (OD_{600}) of 0.8. RNA from each strain was extracted by using the RiboPure bacterial RNA isolation kit (Ambion) according to the manufacturer's guidelines. The total RNA concentration was determined by use of a NanoDrop ND-100 spectrophotometer (NanoDrop Technologies, Wilmington, DE).

Microarrays and data analyses. Analysis of *Escherichia coli* gene expression was performed by using the Affymetrix GeneChip *E. coli* Genome 2.0 array to compare gene expression in strain 86.24 (wild-type enterohemorrhagic *E. coli*), strain SEPT362 (wild type), and strain SEPT362 to those in the Δ*clp* and Δ*hcp* mutants. The GeneChip *E. coli* Genome 2.0 array includes approximately 10,000 probe sets for all 20,366 genes present in the following four strains of *E. coli*: K-12 (laboratory strain MG1655), CFT073 (uropathogenic strain), O157:H7-EDL933 (enterohemorrhagic strain), and O157:H7-Sakai (enterohemorrhagic strain). RNA processing, labeling, hybridization, and slide scanning procedures were performed as described by the manufacturer (Affymetrix gene expression technical manual). Data for Affymetrix GeneChip *E. coli* Genome 2.0 were acquired with a GeneChip Scanner 3000 using GCOS v 1.4 software according to the manufacturer's instructions. Comparisons were performed by using the analysis tools within GCOS v 1.4, by selecting strain SEPT362 as the baseline for comparison. Data were normalized by using MAS 5.0.

RNA slot blots. Bacterial cultures were grown overnight aerobically in LB medium at 37°C, diluted 1:100 in DMEM, and grown at 250 rpm to an OD_{600} of 0.8. RNA from wild-type and mutant samples was extracted by using the RiboPure-Bacteria RNA isolation kit (Ambion) according to the manufacturer's protocol. RNA slot blots were performed by using 1 μg of total RNA in triplicate. The RNA was denatured for 60 min in a solution containing 1 M glyoxal and 0.1 M MOPS (morpholinepropanesulfonic acid) (pH 6.8) at 65°C and applied onto a nylon membrane under a vacuum using a Bio-Rad dot blot apparatus. Wells were then washed with 500 μl of 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). The membranes were cross-linked, hybridized with

TABLE 2. Oligonucleotides

Gene or primer	Sequence
Genes	
Mutagenesis	
clpV	GTTGCGCCAGGCGCTGACGGTGGAGAACTACACAACCGCCCGTTCTGCAGGTGTAGGCTGGGAGCTGCTTC (forward)
	CGCGAGACGGTGTGTTTCTCCTGGAATTCGGACATATTGATGGTGGTCAGCATATGAATATCCTCCTTA (reverse)
hcp	ATGCTCCCATATAATTGACTCATAACTGAAAGAAACTGACTCTTCGGGTTGTGTAGGCTGGGAGCTGCTTC (forward)
	TGATAATCAAGGGCTCATTTCAAGTGGTTGTTCATCACAACCATCCATTGCATATGAATATCCTCCTTA (reverse)
Genetic complementation	
clpV	TACGGATCCACATGATCCAGATTGATCTTCC (forward)
	TAAAGTCGACACTCATAAAACCGTTTCATCCT (reverse)
hcp	TACGGATCCACATGGCGAATTTAATTTATTTAACAC (forward)
	TAAAGTCGACACTTAAAAAAGACGATCTTCCCATAAAC (reverse)
qRT-PCR	
clpV	TGGCCCGTCTGGATAACGT (forward)
	CGTCACTTCCGGTTCAATGA (reverse)
hcp	GCGGAACAATGCTCCCATATA (forward)
	GCGTTCAACCCGAAGAGTCA (reverse)
Primers used for amplification of chloramphenicol cassette	
CmF	ACGGAAGATCACTTCGCA
CmR	GCGTTTAAGGTCAACAATAACTGC

different DNA probes using UltraHyb from Ambion at 42°C, washed first with 2× SSC–0.1% SDS and then with 0.2× SSC–0.1% SDS at 42°C, and exposed to X-ray film. DNA probes for *fimA*, *csgA*, and *rpoA* (negative control) were generated by PCR with *Taq* DNA polymerase amplifying these genes or genes within these operons using the oligonucleotide primers described in Table 2. These probes were labeled by random primer extension using Ready-To-Go DNA labeling beads from Amersham and [α-^{32}P]CTP according to the manufacturer's instructions.

Quantitative real-time RT-PCR assay. Bacterial cultures were grown overnight aerobically in LB medium at 37°C and diluted 1:100 in DMEM grown at 250 rpm to an OD_{600} of 0.8. RNA from three biological samples was extracted by using the RiboPure-Bacteria RNA isolation kit (Ambion) according to the manufacturer's protocol. For comparisons between gene expression in DMEM only and in contact with HeLa cells, HeLa cells were infected with 1×10^7 bacterial cells as well as blank wells (without any HeLa cells); after 3- or 6-h infections, the bacteria were recovered as described below for CFU counts during adherence, and RNA was extracted. To examine the expression of *clpV* during chick infections, we recovered the pericardium and air sac of eight infected animals with strain SEPT362 at 24 h postinfection, and RNA was extracted from these animals. Negative-control PCRs were performed with RNA extracted from the pericardium and air sac from uninfected animals to ensure that there was no cross-reactivity of our primers with the mammalian mRNA message. The primers used for the real-time assays were constructed by using Primer Express v1.5 software (Applied Biosystems) (Table 2). Real-time reverse transcription (RT)-PCR was performed in a one-step reaction by using an ABI 7500 sequence detection system (Applied Biosystems). For each 20-μl reaction mixture, 10 μl 2× SYBR master mix, 0.1 μl Multiscribe reverse transcriptase (Applied Biosystems), and 0.1 μl RNase inhibitor (Applied Biosystems) were added. The amplification efficiency of each primer pair was verified by using standard curves of known RNA concentrations. The *rpoA* (RNA polymerase subunit A) gene was used as the endogenous control. Data collection was performed by using ABI Sequence Detection 1.3 software (Applied Biosystems). Data were normalized to levels of *rpoA* and analyzed by using the comparative critical threshold (C_T) method previously described (75). The expression levels of the target genes at different growth phases were compared by using the relative quantification method (75). Real-time data are presented as fold changes compared to WT levels. Error bars represent the standard deviations (SD) of the $\Delta\Delta C_T$ value (75). Statistical significance was determined by a Student's *t* test. A *P* value of <0.05 was considered significant.

Bacterial adhesion to epithelial cells. For both qualitative and quantitative adhesion assays, *E. coli* strains were evaluated for their abilities to adhere to HeLa cell monolayers by a standard protocol, as previously described, in the absence and in the presence of D-mannose (63). Briefly, the strains were grown in LB medium overnight at 37°C and added in quadruplicate to tissue-cultured cells replenished with fresh DMEM supplemented with 10% fetal bovine serum (FBS) at a concentration of 1×10^7 bacteria per well for 6 h (with a medium change at 3 h) at 37°C in the presence of 5% CO_2. One set of tissue-cultured cells was then washed, fixed, and stained with a 1:40 dilution of Giemsa (Sigma) solution for microscopic evaluation, and the other three sets of cells were washed, lysed with 0.1% Triton X-100 in phosphate-buffered saline (PBS) (pH 7.4), and plated onto LB agar plates for quantification of the numbers of bacterial cells. Adherence data were expressed as CFU per ml of the bacterial inoculum. Statistical differences were expressed as *P* values as determined by a Student's *t* test.

HeLa invasion assays. Epithelial HeLa cells were infected with strain SEPT362, FDP1, or FDP2 at an MOI (multiplicity of infection) of 100:1 for 90 min for bacterium/cell interactions at 37°C in 5% CO_2, as previously described (18, 21, 54, 73). These cells were treated with 40 μg/ml of gentamicin for 90 min to kill extracellular bacteria and lysed with 1% Triton X-100. Bacteria were diluted and plated onto LB plates for CFU determinations (18, 21, 54).

Macrophage assay. J774 murine macrophages were seeded overnight in a 24-well tissue culture plate in a 5% CO_2 incubator at 37°C in DMEM supplemented with glutamine. Wild-type and mutant bacteria were grown in LB broth overnight at 37°C, opsonized with 20% mouse serum at 37°C for 15 min, and added at a concentration of 10^7 CFU/ml, in quadruplicate, to macrophages replenished with fresh DMEM. Nonadherent bacteria were removed by washing three times with 1× PBS and exposed to 50 μg/ml of gentamicin for 90 min to kill any extracellular bacteria. After this time, the plate was washed three times with 1× PBS and incubated for 3 h. The cells were lysed with Triton X-100 (0.1%), and serial 10-fold dilutions were plated in LA to quantify the CFU/ml (10, 19, 45).

FAS test. Fluorescein actin staining (FAS) assays were performed as previously described by Knutton et al. (30). In brief, bacterial cultures grown aerobically overnight in LB medium at 37°C were diluted 1:100 and used to infect confluent monolayers of HeLa cells grown on glass coverslips at 37°C in 5% CO_2. Cells were grown for 6 h at 37°C in 5% CO_2. The coverslips were then washed, permeabilized with 0.2% Triton X-100, and treated with fluorescein isothiocyanate (FITC)-phalloidin to visualize actin accumulation, and propidium iodide was added to stain bacteria. Samples were visualized by immunofluorescence using a Zeiss Axiovert microscope. The entire field of at least six coverslips from each strain was examined, and images of attaching and effacing (AE) lesions were taken.

Chick infection experiments. Chick infection experiments were performed as described previously by Fantinatti et al. (17). Briefly, wild-type and mutant strains were grown overnight into 5.0 ml of LB medium at 37°C, washed, and resuspended into 0.85% (5.0 ml) of sterilized saline solution. A total of 10^9

CFU/ml of each strain was injected into the air sac of 1-day-old male chicks (we used 16 chicks per strain). The groups were observed throughout a 7-day period, and survival was recorded every 12 h.

Statistical analysis. Data are expressed as means ± SD. Bacterial groups in all *in vitro* tests (adherence, survival within macrophages, and invasion into HeLa cells) were compared by using a Student's *t* test for independent samples. Differences were considered significant at a *P* value of <0.05. Statistical analyses were performed with BioEstat, version 5.0.

Microarray data accession number. All array data can be accessed at the Gene Expression Omnibus (GEO) database under accession number GSE21893.

RESULTS

SEPT362 expresses a T6SS. APEC strain SEPT362 was isolated from the liver of a septicemic hen, suggesting that this APEC strain can cause systemic disease. Because the virulence mechanisms of strain SEPT362 are largely unknown, we initially compared the transcriptome of SEPT362 to that of enterohemorrhagic *E. coli* (EHEC) 86-24 to identify the expression of potential virulence genes (array data can be assessed under GEO accession number GSE21893). As previously described for other APEC strains, SEPT362 expressed type 1 and curli fimbriae as well as several iron uptake systems. Of note, SEPT362 expressed T6SS genes at significantly higher levels than did 86-24. These results were confirmed by using quantitative real-time RT-PCR for the *hcp* and *clpV* genes and demonstrate that the transcription of *hcp* and *clpV* is 5-fold and 3.5-fold higher, respectively, in SEPT362 than in 86-24 (Fig. 1A). The presence of these genes in SEPT362 was further confirmed by PCR and DNA nucleotide sequencing.

Because the expression of the T6SS has often been shown to be upregulated in the presence of the host (20), we assessed whether the expression of these genes was upregulated in SEPT362 in the presence of HeLa cells and also during infection of chicks. Figure 1B shows that the expression of both *hcp* and *clpV* increases in the presence of HeLa cells. This expression is enhanced at 3 h and is enhanced even further at 6 h postinfection. The expression of *cplV* was upregulated 58-fold in the air sac and 250-fold in the pericardium of infected animals compared to expression *in vitro* (Fig. 1C). Altogether, these data suggest that SEPT263 expresses a T6SS and that the expression of this system is upregulated within the host.

The T6SS contributes to SEPT362 adherence to epithelial cells. To assess the contribution of the T6SS to *in vitro* virulence phenotypes of SEPT362, *hcp* (strain FDP1) and *clpV* (strain FDP2) nonpolar isogenic mutants were constructed and complemented in *trans* with these genes cloned into a low-copy-number vector (Table 1). Both mutants showed a significant reduction in adherence to HeLa cells of 1 order of magnitude ($P < 0.01$) compared to the wild-type strain, and the complementation of these mutants restored adherence to wild-type levels (Fig. 2). These data suggest that the T6SS is involved in the ability of SEPT362 to adhere to epithelial cells. Several adhesins have been shown to contribute to APEC pathogenesis, and the most studied of these adhesins include type 1, P, and curli fimbriae and temperature-sensitive hemagglutinin (Tsh) (7). Because our transcriptome studies demonstrated that SEPT362 expressed type 1 and curli fimbriae, we investigated the expression of these adhesins in the *hcp* and *clpV* mutants. Both gene arrays (GEO database accession number GSE21893) and RNA slot blot experiments (Fig. 3) demonstrated that while the expression of curli was unchanged

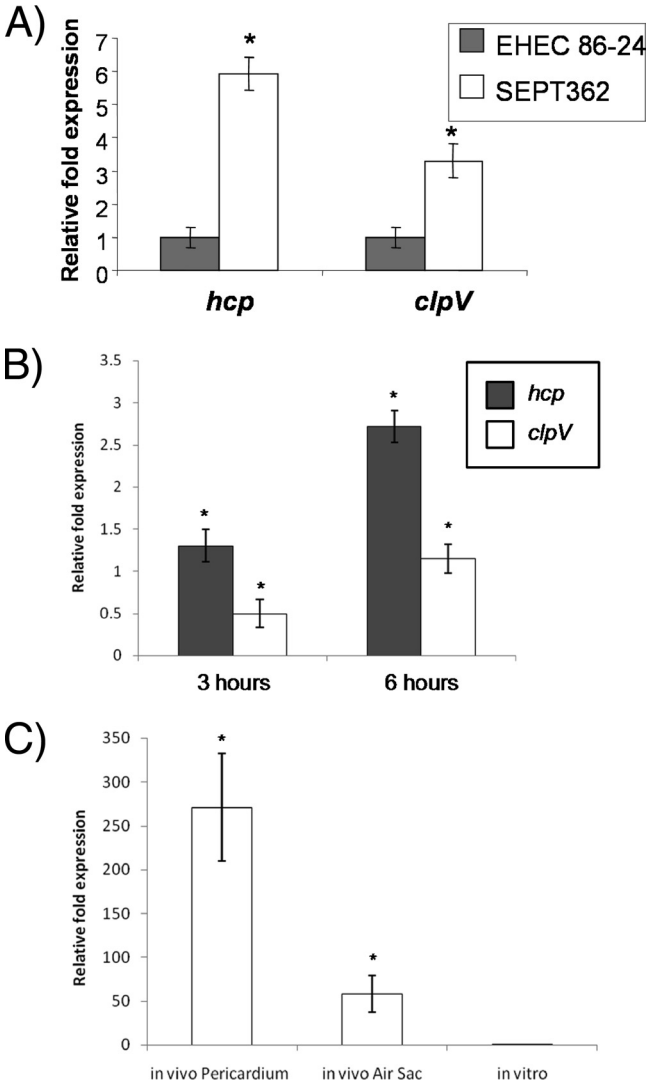

FIG. 1. ASPT362 expresses genes encoding a T6SS. (A) Quantitative real-time RT-PCR of *hcp* and *clpV* genes in the SEPT362 (wild-type strain) compared with EHEC strain 86-24 in DMEM. (B) Quantitative RT-PCR (qRT-PCR) of SEPT362 expressing *clpV* and *hcp* when in contact with HeLa cells for 3 and 6 h in comparison to the absence of these epithelial cells. (C) Expression of *clpV* by SEPT362 in the pericardium and air sac of SEPT362-infected chicks (24 h postinfection) in comparison to expression *in vitro*. Statistical significance was determined by a Student's *t* test. (*, $P < 0.01$).

in these mutants, the expression of type 1 fimbriae was drastically decreased, suggesting that the expressions of the T6SS and type 1 fimbriae are coordinated (Fig. 3). Fimbrial genes are sometimes found within loci encoding T6SSs, and because the genome of SEPT362 has not been sequenced, we do not know whether the genes encoding the type 1 fimbriae are clustered with the T6SS genes in this strain. The fact that fimbrial genes can be adjacent to the T6SS genes raises the possibility that the lack of expression of type 1 fimbriae in our T6SS mutants could be due to polar effects in these mutants. However, we ruled this possibility out because these mutants are nonpolar deletions constructed using λ red, the "scars" have been sequenced to ensure that no polar effects would

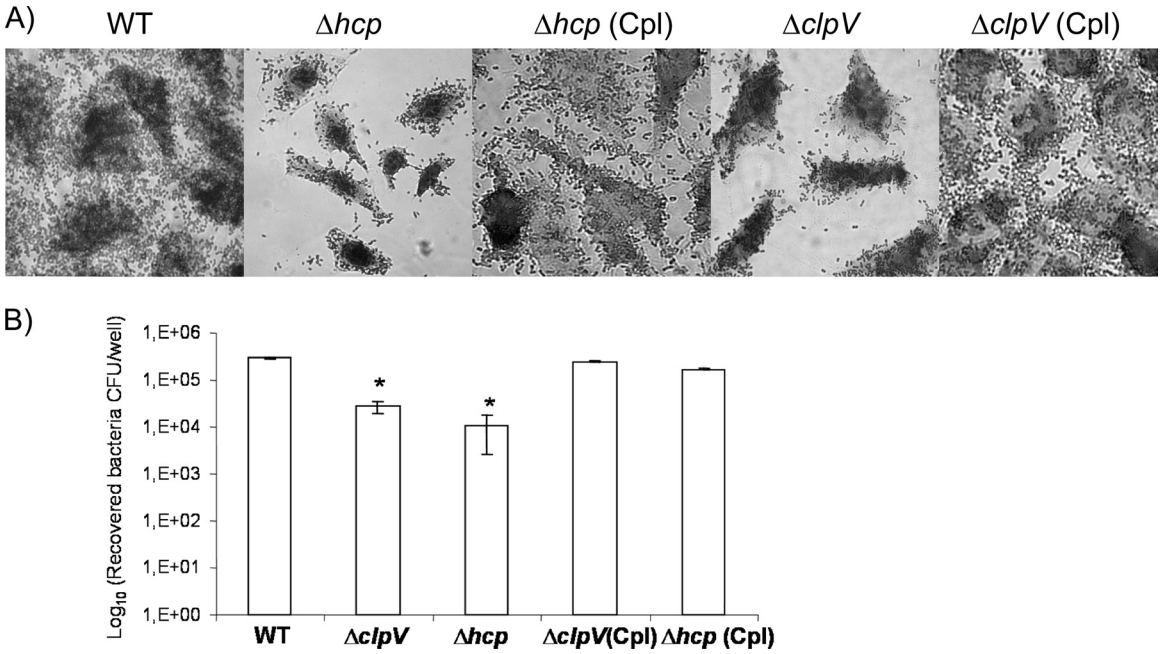

FIG. 2. The T6SS is involved in SEPT362 adhesion to HeLa cells. (A) Giemsa stain of strain SEPT362 and the Δ*hcp*, Δ*clpV*, and complemented (Cpl) strains adhering to HeLa cells. (B) Quantification of adhesion of strain SEPT362 and the Δ*hcp*, Δ*clpV*, and complemented strains to HeLa epithelial cells. Statistical significance was determined by a Student's *t* test based on comparisons with strain SEPT362 (wild type) (*, $P < 0.01$).

occur in adjacent genes, and the effects in adhesion can be complemented with the *clpV* or *hcp* gene in *trans*.

Because the decreased adhesion of the T6SS mutants could be due to the lower expression level of type 1 fimbriae, we repeated the adhesion tests in the presence of D-mannose. It has been well documented that type 1 fimbriae bind to mannose oligosaccharides and that in the presence of D-mannose, type 1 fimbria-dependent adherence is inhibited (29, 36, 42, 52, 69). We reasoned that if the adherence defect of the *clpV* and *hcp* mutants (Fig. 2) was due to a decreased expression on type 1 fimbriae (Fig. 3) in the presence of mannose, the mutants would adhere as well as the WT to HeLa cells. Indeed, in the presence of mannose the mutants adhered to HeLa cells at levels indistinguishable from those of the wild type (Fig. 4), supporting the hypothesis that the defect in adherence was due to the lower level of expression of type 1 fimbriae by the T6SS mutants.

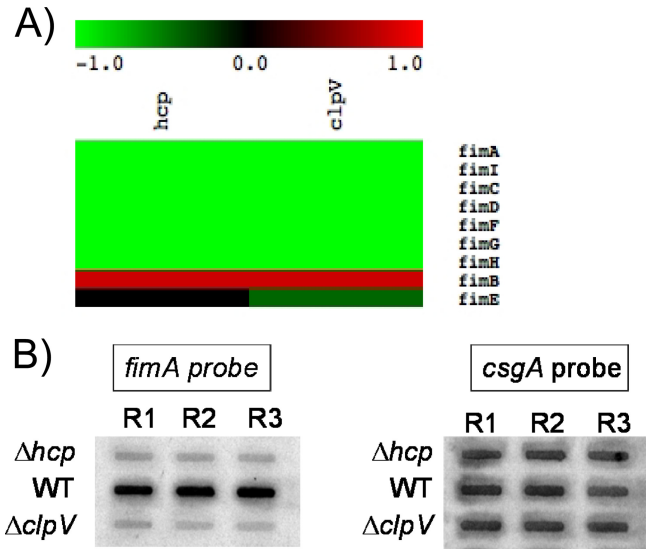

FIG. 3. The T6SS alters type 1 fimbria expression in SEPT362. (A) Heat map depicting expression of the *fim* genes in the *hcp* and *clpV* mutants compared to the wild type (green indicates that transcription in the mutants is decreased compared to that in the WT, and red indicates that transcription is increased in the mutants compared to that in the WT). (B) RNA slot blots depicting the expression of *fimA* and *csgA* in the wild type (WT) and the *hcp* and *clpV* mutants. R1, R2, and R3 refer to three biologically different RNA samples tested.

The T6SS contributes to SEPT362 invasion of epithelial cells. Because some strains of APEC have been shown to also invade epithelial cells (23), and the T6SS affects actin polymer-

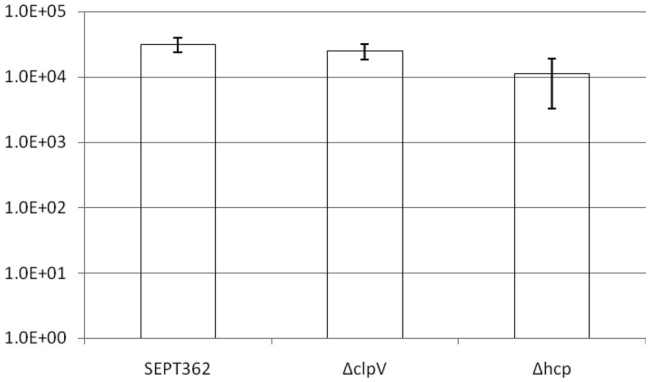

FIG. 4. Quantification of the adhesion of SEPT362 and the Δ*hcp*, Δ*clpV*, and complemented strains to HeLa epithelial cells in the presence of D-mannose.

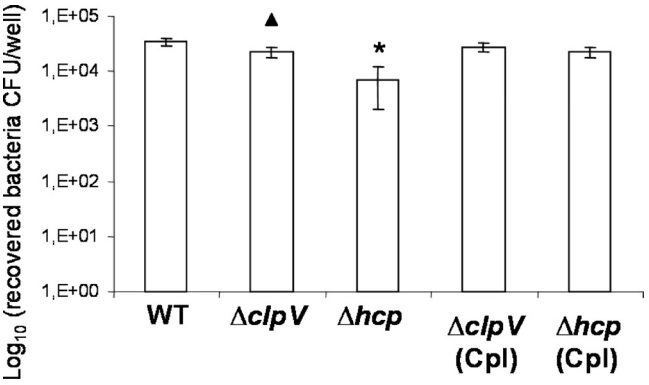

FIG. 5. Invasion of HeLa epithelial cells by SEPT362 (WT strain) and the Δ*hcp*, Δ*clpV*, and complemented strains. Statistical significance was determined by a Student's *t* test based on comparisons with strain SEPT362 (*, $P < 0.01$; ▲, $P > 0.01$ [not significant]).

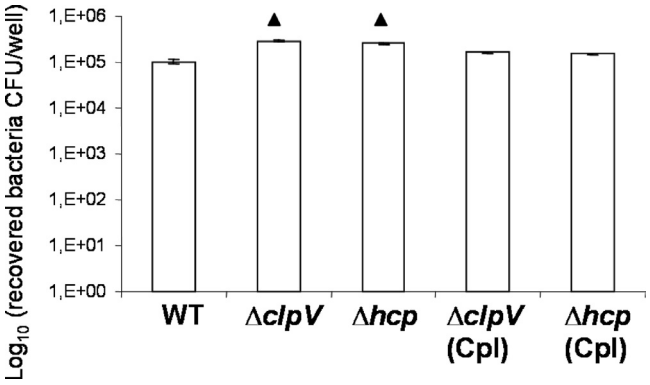

FIG. 7. Replication of the WT, Δ*hcp*, Δ*clpV*, and complemented strains in J774 macrophages. Statistical significance was determined by a Student's *t* test based on comparisons with strain SEPT362 (▲, $P > 0.01$ [not significant]).

ization (35, 37, 44, 56, 57), we next assessed whether strain SEPT362 was invasive as well as the potential contribution of the T6SS to this phenotype. Strain SEPT362 was able to efficiently invade HeLa cells (Fig. 5), and the T6SS played a small but significant role in this phenotype. The *hcp* mutant depicted a significant decrease of 1 order of magnitude in HeLa cell invasion compared to the WT, which could be complemented with this gene provided *in trans*, while the *clpV* mutant invaded HeLa cells as efficiently as did the WT (Fig. 5).

The best-characterized effector of the T6SS is VgrG, which has been shown to cross-link actin (56); we have sequenced the *vgrG* gene of SEPT362, and it has an actin modification C-terminal extension (see the supplemental material). Inasmuch as actin rearrangement is central to bacterial invasion of epithelial cells (22), and the T6SS contributes to the invasion of epithelial cells by SEPT362 (Fig. 5), actin staining tests to assess cytoskeleton rearrangements of infected epithelial cells were performed. SEPT362 induced the formation of filopodia and ruffle-like structures on HeLa cells (Fig. 6). Consistent with its decreased ability to invade epithelial cells, cells infected with *hcp* depicted markedly decreased levels of cytoskeleton rearrangement. However, while the *clpV* mutant still invaded epithelial cells to levels similar to those of the wild type (Fig. 5), it also produced decreased levels of cytoskeleton rearrangements on epithelial cells (Fig. 6).

Hcp and ClpV are not involved in intramacrophage replication. Many bacteria that cause systemic disease can survive phagocytosis and replicate within macrophages (23). Because SEPT362 causes a systemic septicemic disease, we investigated whether this strain could replicate within macrophages. Figure 7 shows that SEPT362 can replicate within macrophages and that Hcp and ClpV do not contribute to this pathogenic trait.

The T6SS is important for the virulence of SEPT362 *in vivo*. Because T6SS mutants showed decreased adherence to and invasion of epithelial cells, the contribution of this specialized secretory apparatus to pathogenesis *in vivo* was investigated. For this purpose, chicks were infected with wild-type SEPT362, the *hcp* and *clpV* mutants, and complemented strains (Fig. 8). Within 24 h, only 43.75% of the chicks infected with SEPT362 survived, and by day 2 postinfection, just 12.5% of these animals remained alive until day 7, when they were sacrificed. For chicks infected with the Δ*hcp* strain, we observed that 87.5% survived by day 2, 62.5% of chicks survived by day 3, 56.25% survived by day 4, 43.75% survived by day 5, and 25% remained alive until the end of the experiment. For animals infected with the Δ*clpV* strain, we observed that 93.75% survived on the first day, 68.75% survived by day 3, 62.5% survived by day 4, 50% survived by day 6, and 43.75% survived by day 7. These data indicate that *hcp* and *clpV* mutants are attenuated for pathogenesis in chicks and that the *clpV* mutant is more attenuated than the *hcp* mutant, consistent with the observation that in addition to diminished adherence to epithelial cells (Fig. 2), the *clpV* mutant also has a decreased level of invasion of epithelial cells (Fig. 5). The complementation of these genes

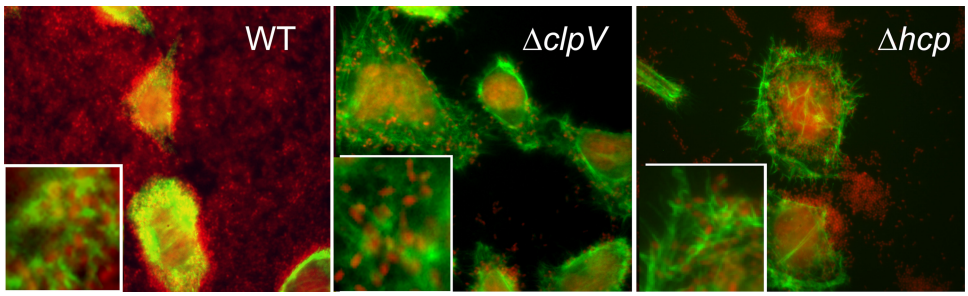

FIG. 6. FAS test of the WT, Δ*hcp*, and Δ*clpV* strains in HeLa cells. Actin was stained with FITC-labeled phalloidin (green), and the bacteria and HeLa cell nuclei were stained with propidium iodide (red).

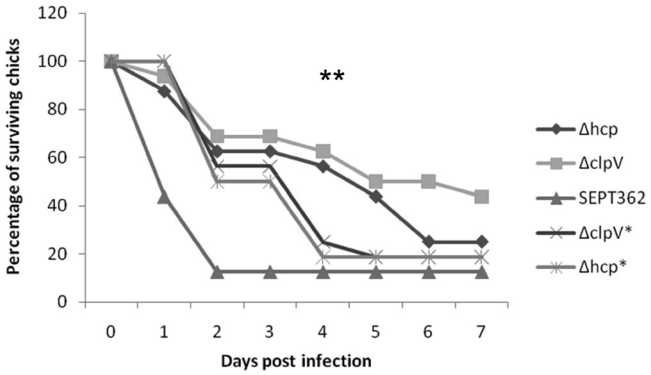

FIG. 8. Survival of chicks infected with 10^9 CFU/ml bacteria (16 chicks per strain tested). Statistical significance was determined by a Student's *t* test (**, $P < 0.05$), based on comparisons with strain SEPT362 (wild type). *, complemented strains.

in *trans* partially rescued these phenotypes, and the complemented strains were more pathogenic than the mutants but not as virulent as the wild-type strain. Because these mutants were complemented with these genes cloned into plasmids, the different infection kinetics of the complements compared to the wild type could be due to differential gene dosages as well as plasmid maintenance during infection.

DISCUSSION

The interaction between a bacterial pathogen and eukaryotic cells depends on signaling through proteins and the delivery of toxins, bacterial effectors, chemicals, and cell surface adhesins to mediate specific attachment to the host membrane (2). Bacterial adherence to host cells is generally the first step in infections caused by *E. coli* and is important during the initial stages of host colonization (2).

Infections with APEC cause systemic disease, resulting in significant economic losses to the poultry industry worldwide. Recently, it has been appreciated that APECs are closely related to other ExPECs that cause diseases in humans (46, 47), suggesting that there is a potential risk for the zoonotic transmission of these strains. The most studied virulence factors of APEC are adhesins, iron acquisition systems, hemolysins, factors involved in serum resistance and resistance to phagocytosis, as well as toxins and cytotoxins (40, 41, 76). Here we showed for the first time that *hcp* and *clpV* (T6SS genes) can influence the expression of type 1 fimbriae, are involved in the invasion of and adherence to HeLa cells, and are important for pathogenicity in an *in vivo* model, suggesting an important role for the T6SS in APEC pathogenesis (Fig. 1 to 8). Indeed, the expression of T6SS genes is often correlated with the presence of host cells (8, 12, 68), the levels of expression of *clpV* and *hcp* are increased in the presence of HeLa cells, and the level of expression of *clpV* is dramatically increased during infection of chicks (Fig. 1).

Type 1 pili are filamentous bacterial appendages that promote bacterial adherence to eukaryotic cells. The gene cluster responsible for type 1 pilus formation is composed of nine genes (5, 10, 24, 28, 32, 38, 48–50, 52, 60, 73). Through microarray analyses and RNA slot blot assays, we showed that the expression of type 1 fimbriae was decreased in the Δ*hcp* and Δ*clpV* mutants (Fig. 3). These results suggest that the diminished capability of the adhesion of these mutants to epithelial cells may be a result of the decrease in the level of expression of type 1 fimbriae (Fig. 2 and 4). However, our transcriptome and RNA slot blot expression analyses show that SEPT362 also expresses other adhesins, such as the curli fimbriae (Fig. 3). Although the T6SS influences the expression of type 1 fimbriae, it does not alter the expression of curli, suggesting that the association of T6SS assembly and type 1 fimbriae is somewhat specific. Since it was previously reported that changes in membrane proteins lead to changes in outer membrane composition (53), we suggest that these alterations of gene expression could happen due to remodeling of the cell surface when regular membrane structures are altered, in an attempt to "compensate" for the lack of a surface structure during the pathogenesis process. It is also worth noting that type 1 fimbriae play an important role in biofilm formation in *E. coli* (1, 26, 65, 66), that the expression of the T6SS was reported to be enhanced during biofilm formation, and that the T6SS is also involved in biofilm formation (4, 8, 16, 71, 74). Because mutations in the T6SS decrease the level of expression of type 1 fimbriae, the role of the T6SS in biofilm formation could be due to decreased fimbrial gene expression.

The invasion of epithelial cells was significantly decreased in the Δ*hcp* mutant and unaltered in the Δ*clpV* mutant (Fig. 5). However, both mutants (Fig. 6) induced decreased cytoskeleton rearrangements on epithelial cells compared to the wild type. The decreased cytoskeleton rearrangement observed for the T6SS mutants could be a result of the decreased level of expression of type 1 fimbriae, which was shown previously to contribute to UPEC invasion of bladder cells (48). Alternatively, it could be due to a decreased delivery of the VgrG effector, which has been shown to cross-link actin (56). Although SEPT362 can efficiently replicate within macrophages, this phenotype does not seem to involve *hcp* and *clpV* (Fig. 7).

The T6SS has been associated with virulence and host associations in several bacterial species. In *V. cholerae* and *P. aeruginosa* the T6SS is required for associations with *Dictyostelium discoideum* (55, 57), and the *P. aeruginosa* T6SS likely contributes to the pathogenesis of this bacterium in cystic fibrosis patients (44). The Hcp homolog is secreted into the host cells via the T6SS of *Aeromonas hydrophila* and contributes to virulence in a septicemic mouse model (72). Hcp has also been shown to be secreted by *Burkholderia mallei* and to be required for virulence in a hamster model of infection (64). Additionally, in *Edwardsiella tarda* the T6SS is essential for virulence in fish (77). Here we show that the T6SS contributes to the pathogenicity of APEC in chicks, adding this specialized secretory pathway to the varied repertoire of APEC virulence traits. Future studies will further define the role of the T6SS in APEC pathogenesis and delve into the molecular mechanisms associated with the assembly and function of this system. The broad association of the T6SS with bacterial pathogenesis makes the T6SS an attractive target for therapeutics and vaccine development.

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