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Received 24 March 2011 Revised 1 June 2011 Accepted 19 July 2011

Characterization of lcmF of the type VI secretion system in an avian pathogenic *Escherichia coli* (APEC) strain

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The intracellular multiplication factor (IcmF) protein is a component of the recently described type VI secretion system (T6SS). IcmF has been shown to be required for intra-macrophage replication and inhibition of phagosome-lysosome fusion in Legionella pneumophila. In Vibrio cholerae it is involved in motility, adherence and conjugation. Given that we previously reported that two T6SS genes (hcp and clpV) contribute to the pathogenesis of a septicaemic strain (SEPT362) of avian pathogenic Escherichia coli (APEC), we investigated the function of IcmF in this strain. Further elucidation of the virulence mechanisms of APEC is important because this pathogen is responsible for financial losses in the poultry industry, and is closely related to human extraintestinal pathogenic E. coli (ExPEC) strains, representing a potential zoonotic risk, as well as serving as a reservoir of virulence genes. Here we show that an APEC icmF mutant has decreased adherence to and invasion of epithelial cells, as well as decreased intra-macrophage survival. The icmF mutant is also defective for biofilm formation on abiotic surfaces. Additionally, expression of the flagella operon is decreased in the icmF mutant, leading to decreased motility. The combination of these phenotypes culminates in this mutant being altered for infection in chicks. These results suggest that IcmF in APEC may play a role in disease, and potentially also in the epidemiological spread of this pathogen through enhancement of biofilm formation.

INTRODUCTION

Avian pathogenic *Escherichia coli* (APEC) strains are a frequent cause of extraintestinal diseases, with respiratory or systemic infections being responsible for large financial losses for the poultry industry worldwide (Barnes *et al.*, 2003; Gross, 1994; Gyles, 1993). The most severe systemic disease, colisepticaemia, is characterized by pericarditis, perihepatitis, air-sacculitis, synovitis and peritonitis (La Ragione & Woodward, 2002). Furthermore, APEC has

Abbreviations: APEC, avian pathogenic *E. coli*; EHEC, enterohaemorrhagic *E. coli*; ExPEC, extraintestinal pathogenic *E. coli*; qRT-PCR, quantitative real-time RT-PCR; T6SS, type VI secretion system; UPEC, uropathogenic *E. coli*.

The GEO accession number for the microarray data associated with this paper is GSM544428.

been reported to share important virulence traits with uropathogenic *E. coli* (UPEC), representing a possible zoonotic risk or a reservoir of virulence genes for these and other extraintestinal *E. coli* (ExPEC) strains (Zhao *et al.*, 2009). Several virulence genes have been described in APEC (Dho-Moulin & Fairbrother, 1999; Mellata *et al.*, 2003; Yaguchi *et al.*, 2007; Zhao *et al.*, 2009); however, the virulence repertoire in many strains remains largely uncharacterized.

An important bacterial virulence trait is the delivery of proteins and toxins to host cells through specialized secretion systems (Bingle *et al.*, 2008; Cascales, 2008; Filloux *et al.*, 2008; Pukatzki *et al.*, 2009). These secretion systems release virulence factors into the environmental milieu or directly within the eukaryotic cells. Among the described secretion systems, the type VI secretion system

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(T6SS) has recently been reported in a number of bacterial species (Shrivastava & Mande, 2008), and it has been suggested that it plays an important role in virulence of several pathogens (Gray *et al.*, 2002; Parsons & Heffron, 2005; Schell *et al.*, 2007), including APEC (de Pace *et al.*, 2010).

T6SS components are encoded within clusters of genes that were initially characterized as IAHP (IcmF-associated homologous proteins), because all of them contained a gene encoding an IcmF-like (intracellular multiplication factor) component that was first reported and described in Legionella pneumophila, where it is currently known to be part of a type IV secretion system (Solomon et al., 2000). The IcmF protein in L. pneumophila is required for replication in macrophages, phagocytosis, inhibition of phagosome-lysosome fusion during infection and apoptosis (Purcell & Shuman, 1998; Zink et al., 2002). This protein, which is localized in the inner membrane (Sexton et al., 2004), contains several transmembrane domains and a putative Walker A nucleotide-binding motif, and is known to be part of the process to deliver proteins into target cells. Additionally, it was previously shown that Vibrio cholerae icmF is involved in motility, adherence to epithelial cells, and increased conjugation frequency (Das et al., 2002).

Since APEC strains encode a T6SS and contain an *icmF* gene, we constructed an *icmF* mutant in a septicaemic APEC strain and analysed its effects on virulence-related phenotypes *in vitro*, as well as attenuation in an *in vivo* chick infection model. This mutant showed decreased ability to adhere to and invade HeLa cells, was non-motile, and showed reduced biofilm formation. These results suggest that the *icmF* gene in APEC may contribute to its pathogenesis.

METHODS

Bacterial strains and growth conditions. APEC strain SEPT362 (OR:H10) (streptomycin, tetracycline and ampicillin resistant) was isolated from the liver of a broiler with clinical signs of septicaemia, and belongs to the bacterial collection of the Bacterial Molecular Biology Laboratory, Insitute of Biology, Campinas State University

(Unicamp). Strains were grown aerobically at 37 °C, using Dulbecco's Modified Eagle's Medium (DMEM; Invitrogen) or Luria–Bertani (LB) medium (Fritsch *et al.*, 1989). Antibiotics were added at the following concentrations: 100 μg ampicillin ml⁻¹, 30 μg chloramphenicol ml⁻¹, 50 μg kanamycin ml⁻¹ and 25 μg tetracycline ml⁻¹. Recombinant DNA and molecular biology techniques were performed as described by Sambrook *et al.* (1989). All strains and plasmids used in this study are listed in Table 1.

Construction of the icmF mutant and complemented strain.

The $\Delta icmF$ strain was constructed using the λ Red system (Datsenko & Wanner, 2000). Briefly, oligonucleotide primers specific to the kanamycin (Km) cassette flanked by 50 nt extensions homologous to the regions adjacent to the target gene (icmF) were used to amplify the kanamycin cassette from plasmid pKD4. The purified PCR product was electroporated into strain SEPT362 containing the λ Red recombinase plasmid pKD46C [pKD46 modified by the insertion of a chloramphenicol cassette amplified from plasmid pACYC184 (de Pace et al., 2010)], and the transformed bacterial cells were plated and grown on LB agar containing streptomycin and kanamycin at 37 °C. The non-polar mutant was resolved using plasmid pCP20 (Datsenko & Wanner, 2000). Deletion of the icmF gene in strain FDP7 was confirmed by PCR and DNA sequencing. To complement the $\Delta icmF$ strain, PCR-amplified icmF was cloned into the BamHI and SalI sites of plasmid pACYC184, generating the plasmid pFDP9. This plasmid was transformed into the FDP7 strain, generating the complemented strain FDP8. All primers used in this work are listed in Table 2.

RNA extraction. Cultures of enterohaemorrhagic *E. coli* (EHEC) strain 86-24, wild-type SEPT362 and FDP7 ($\Delta icmF$) APEC strains were grown aerobically in LB medium at 37 °C overnight, diluted 1:100 in Dulbecco's Modified Essential Medium (DMEM; Invitrogen) and grown at 37 °C and 250 r.p.m. to an OD₆₀₀ of 0.8. RNA from each strain was extracted using the RiboPure bacterial RNA isolation kit (Ambion), following the manufacturer's guidelines. Total RNA concentration was determined with a NanoDrop ND-100 spectrophotometer (NanoDrop Technologies).

Microarrays and data analysis. The *E. coli* Genome 2.0 gene chip, which contains the complete genomes of four *E. coli* strains (non-pathogenic *E. coli* K-12 strain MG1655, UPEC strain CFT073, and EHEC O157: H7 strains EDL933 and Sakai – http://www.affymetrix.com/products/arrays/specific/ecoli2.affx) was employed to analyse and compare gene expression between strains SEPT362 and FDP7. RNA processing, labelling, hybridization and slide scanning procedures were performed as described in the *Affymetrix Gene Expression Technical Manual* (http://www.affymetrix.com/support/technical/manual/expression_manual.affx). Data of Affymetrix GeneChip *E. coli* Genome 2.0 were acquired by GeneChip Scanner 3000 using the software GCOS v. 1.4 according to the manufacturer's instructions.

Table 1. Strains and plasmids

Strain or plasmid	Relevant genotype	Source or reference
SEPT362	APEC strain isolated from a septicaemic broiler	This study
FDP7	icmF mutant	This study
FDP8	$\Delta icmF$ with pFP9	This study
pACYC184	Cloning vector	New England Biolabs
pKD4	pANTSγ derivative containing FRT-flanked kanamycin resistance	Datsenko & Wanner (2000)
pKD46	λ Red recombinase expression plasmid	
pKD46C	pKD46 with a chloramphenicol cassette insertion	This study
pKDP20	TS replication and thermal induction of FLP synthesis	Datsenko & Wanner (2000)
pFDP9	icmF in pACYC184	This study

Table 2. Oligonucleotides

Primer	Sequence	
Primers used for mutagenesis		
Forward	TACGGATCCACATGGTCATCGGCCCGGC	
Reverse	TAAAGTCGACACTCAGTACAGTGTGTCTGAC	
Primers used for genetic complementation		
Forward	TACGGATCCACATGGTCATCGGCCCGGC	
Reverse	TAAAGTCGACACTCAGTACAGTGTGTCTGAC	
Primers used for qRT-PCR		
Forward	GACATGGGTGGATCGAATGAA	
Reverse	TGTGAGTCTGGGTGACCATCA	
Primers used for amplification of Cm cassette		
Forward	ACGGAAGATCACTTCGCA	
Reverse	GCGTTTAAGGTCAACAATAACTGC	

Comparisons were performed using the analysis tools within GCOS v. 1.4, selecting strain SEPT362 as the baseline for comparison. Data were normalized using MAS 5.0. All array data can be accessed using the GEO database number GSM544428.

Quantitative real-time RT-PCR (qRT-PCR). Bacterial cultures were grown overnight aerobically in LB at 37 °C and diluted 1:100 in DMEM, then grown at 250 r.p.m. to OD_{600} 0.8. RNA from three biological samples was extracted using the RiboPure-Bacteria RNA isolation kit (Ambion), following the manufacturer's instructions. Primers are listed in Table 2. Real-time RT-PCR was performed in a one-step reaction using an ABI 7500 sequence detection system (Applied Biosystems). For each 20 μl reaction, 10 μl 2 × SYBR Master Mix, 0.1 µl Multi-scribe reverse transcriptase (Applied Biosystems), and 0.1 µl RNase inhibitor (Applied Biosystems) were added. Amplification efficiency of each primer pair was verified using standard curves of known RNA concentrations. The rpoA (RNA polymerase subunit A) gene was used as the endogenous control. Data collection was performed using the ABI Sequence Detection 1.3 software (Applied Biosystems). Data were normalized to levels of rpoA and analysed using the comparative critical threshold (C_t) method previously described (Walters & Sperandio, 2006). The expression level of the target genes was compared using the relative quantification method. Real-time data are presented as fold change compared to wild-type levels. Error bars represent the standard deviation of the $2^{-\Delta\Delta C_t}$ value. Statistical significance was determined by Student's t test. A P-value of < 0.05 was considered significant.

Bacterial adhesion to epithelial cells. *E. coli* strains were evaluated for their ability to adhere to HeLa cell monolayers by a standard protocol as previously described (Scaletsky *et al.*, 1984). 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 at a concentration of 1×10^7 c.f.u. per well, and then incubated for 6 h (with a medium change at 3 h) at 37 °C in the presence of 5 % $\rm CO_2$. One set of tissue-cultured cells was then washed, fixed, and stained with 1:40 Giemsa (Sigma) solution for microscopic evaluation, and the other three sets were washed, lysed with 0.1 % Triton X-100 in PBS (pH 7.4), and plated on LB agar plates for quantification of c.f.u. Adherence data were expressed as c.f.u. per ml of the bacterial inoculum. The significance of differences in adhesion was expressed as *P*-values as determined by *t*-test analysis.

HeLa invasion assay. Epithelial HeLa cells were infected with strain SEPT362, FDP7 or FDP8 at an m.o.i. of 100:1 for 90 min at 37 °C,

5 % CO₂, as previously described (Fierer *et al.*, 1993; Finlay *et al.*, 1991; Pfeifer *et al.*, 1999; Vidotto *et al.*, 1990). These cells were treated with 40 μg gentamicin ml⁻¹ for 90 min to kill extracellular bacteria, and lysed with 1 % Triton X-100. Bacteria were diluted and plated on LB agar for c.f.u. determination (Fierer *et al.*, 1993; Finlay *et al.*, 1991; Pfeifer *et al.*, 1999).

Analysis of biofilm formation using crystal violet staining. The ability of strains SEPT362, FDP7 and FDP8 to form biofilms was assessed in triplicate using DMEM, according to Christensen et al. (1985). Overnight bacterial cultures grown under static conditions were inoculated into fresh medium in a 1:100 dilution in 24-well cellcultures plates (TPP) with and without glass coverslips, in a final volume of 1000 μl. These plates were incubated at 37 °C in a 5 % CO₂ atmosphere for 24 h. At the end of the incubation time, the culture medium was discarded, and the wells washed three times with PBS. Cells were fixed with 1000 μ l 75% ethanol, and washed three times with $1 \times PBS$ to remove the ethanol. The cells were then stained with 0.5% crystal violet for 5 min. After four PBS washings, the plates were dried and the crystal violet was solubilized by the addition of 1000 μl 95 % ethanol to each well. After 2 min at room temperature, 150 µl of the solution was transferred to a microtitre plate, and the absorbance was determined in a spectrophotometer at 570 nm.

Macrophage assays. 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 in a concentration of 10^7 , in quadruplicate, to macrophages replenished with fresh DMEM. Non-adherent bacteria were removed by washing three times with PBS and treated with gentamicin for 90 min to kill any extracellular bacteria. The plate was then washed three times with PBS and incubated for 3 h. The cells were lysed with Triton X-100 (1%) and serial dilutions plated for c.f.u. determination.

Motility assays. Assays were performed as previously described (Clarke & Sperandio, 2005). Briefly, static overnight cultures were stabbed on LB soft agar (0.3% agar) motility plates and incubated at 37 °C; motility haloes were measured at 12 h.

Chick infection experiments. Chicks (1-day-old male commercial broiler chickens) were infected with wild-type and mutant strains as previously described by de Pace *et al.* (2010). Briefly, wild-type and mutant strains were grown overnight in LB medium, at 37 $^{\circ}$ C, washed and resuspended in saline solution. A total of 10 9 c.f.u. ml $^{-1}$ of each strain was injected into the air sac of groups of 16 1-day-old male chicks. The groups were observed throughout a 7 day period and survival was recorded every 12 h.

Statistical analysis. Data are expressed as means \pm SD. Bacterial groups were compared using Student's *t*-test for independent samples and differences were considered significant at P<0.05. For chick infection assays, the chi-squared test was used. Statistical analyses were performed with BioEstat version 5.0.

RESULTS

SEPT362 expresses icmF

The APEC strain SEPT362 was isolated from the liver of a septicaemic broiler. Previous transcriptome analyses of SEPT362 compared to the EHEC strain 86-24 revealed that SEPT362 expresses the T6SS genes, including *icmF*, at

significantly higher levels than strain 86-24 (de Pace *et al.*, 2010). These results were confirmed using qRT-PCR for the *icmF* gene and demonstrate that transcription of *icmF* is twofold higher in SEPT362 when compared to 86-24 (data not shown). The presence of this gene in SEPT362 was further confirmed by PCR and DNA nucleotide sequencing.

IcmF is involved in adherence to HeLa cells

Because IcmF has been previously described to be involved in adherence to epithelial cells by V. cholerae (Das et al., 2002), and other components of the T6SS in APEC (Hcp and ClpV) have also been shown to contribute to APEC adherence to HeLa cells (de Pace et al., 2010), we first assessed whether IcmF is also necessary for APEC to adhere to these cells. The adherence of the $\Delta icmF$ strain to HeLa cells was decreased by one order of magnitude (P < 0.01) compared to the wild-type strain, and this phenotype was restored upon complementation with the icmF gene on a low-copy-number plasmid (wild-type adhesion was 3×10^5 $+3 \times 10^4$ c.f.u.; $\triangle icmF$ adhesion was $4.5 \times 10^4 + 6 \times 10^3$ c.f.u.; and the adhesion of the complemented strain was $2.8 \times 10^5 \pm 3 \times 10^4$ c.f.u.) (Fig. 1). These results suggest that IcmF also contributes to APEC adhesion to HeLa cells. Because the contribution of other components from the T6SS, namely ClpV and Hcp, to adhesion to HeLa cells was solely due to the diminished expression of type 1 fimbriae in these mutants (de Pace et al., 2010), we assessed whether expression of these fimbriae was also decreased in the icmF mutant. qRT-PCR showed that expression of fimA (the gene encoding the major subunit of type 1 fimbriae) is identical between the wild-type and the $\Delta icmF$ strains (data not shown), suggesting that IcmF's contribution to adhesion to HeLa cells is independent of type 1 fimbriae.

Biofilm formation is influenced by the icmF gene

Given that we observed that SEPT362 adheres to polystyrene and epithelial cells, with a pattern that resembles biofilm formation, and that this adherence is decreased in the *icmF* mutant (Fig. 1), we assessed whether this mutant showed an altered ability to form biofilms on

abiotic surfaces. The icmF mutant does not show any growth defects when compared to the wild-type (data not shown). Using the crystal violet biofilm test (Watnick & Kolter, 1999), we observed that the $\Delta icmF$ strain was defective in biofilm formation on polystyrene and glass compared to the wild-type strain (Fig. 2). On a polystyrene surface the ΔicmF strain formed 80 % less biofilm than the wild-type (Fig. 2a), and on a glass surface, the $\Delta icmF$ completely lost its ability to form a biofilm (Fig. 2b), which indicates that icmF gene stimulates biofilm formation. The complemented strain restored 50 % of biofilm formation on polystyrene, and completely restored biofilm formation on glass. The differential complementation observed on these two surfaces could be a result of the enhanced gene dosage of icmF in the complemented strain affecting expression of sets of genes necessary for biofilm formation on polystyrene, but not on glass.

IcmF is important for invasion of epithelial cells

We have also shown that APEC strain SEPT362 can invade HeLa cells, and that Hcp, but not ClpV, contributes to this phenotype (de Pace *et al.*, 2010). Because of the differential contribution of distinct components of SEPT362 T6SS to the invasion of epithelial cells, we also investigated whether IcmF played a role in this phenotype. The $\Delta icmF$ strain showed a significant decrease of one order of magnitude for invasion of HeLa cells when compared to the wild-type and complemented strains (P < 0.01) (invasion was $3.5 \times 10^4 \pm 6 \times 10^3$ c.f.u. for the wild-type; $2 \times 10^3 \pm 1 \times 10^2$ c.f.u. for $\Delta icmF$; and $2.2 \times 10^4 \pm 4 \times 10^3$ c.f.u. for the complemented strain), suggesting that both Hcp and IcmF contribute to invasion of epithelial cells, while ClpV is not involved in this phenotype.

Replication and viability inside macrophages

Strain SEPT362 causes septicaemia in chicks, and can replicate within macrophages (de Pace *et al.*, 2010). However, our previous observations suggested that the T6SS components ClpV and Hcp were not involved in intra-macrophage replication (de Pace *et al.*, 2010). Given that we have observed that different T6SS components play

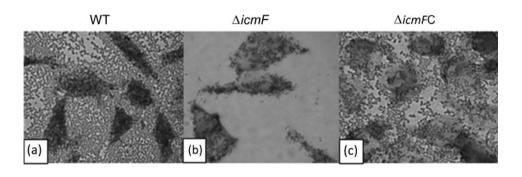


Fig. 1. Adhesion to HeLa cells of (a) wild-type SEPT362, (b) ΔicmF and (c) ΔicmF complemented strain (ΔicmFC).

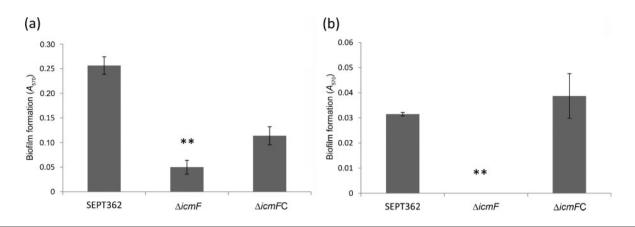


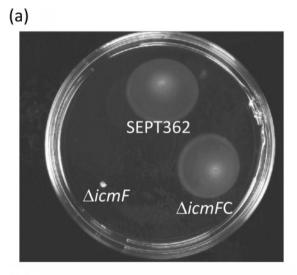
Fig. 2. Biofilm formation. (a) Cells were grown for 24 h in polystyrene plates containing DMEM. (b) Cells were grown for 24 h in a 24-well plate, with glass coverslips, containing DMEM. Biofilm formation was assessed by crystal violet staining. **, *P*<0.05.

diverse roles in invasion of epithelial cells as described above and reported by de Pace *et al.* (2010), we assessed whether IcmF was involved in intra-macrophage survival. Our results showed that the intra-macrophage survival of the $\Delta icmF$ strain in J774 macrophages was decreased by one order of magnitude compared to the wild-type and complemented strains (P<0.01) (survival was $1 \times 10^5 \pm 9 \times 10^3$ c.f.u. for the wild-type; $3 \times 10^4 \pm 5 \times 10^3$ c.f.u. for $\Delta icmF$; and $7.8 \times 10^4 \pm 8 \times 10^3$ c.f.u. for the complemented strain). These data suggest that IcmF, unlike Hcp and ClpV, is involved in efficient intra-macrophage replication of strain SEPT362, further highlighting that different components of the T6SS in this strain differentially contribute to distinct phenotypes.

IcmF is involved in motility

The above results show that IcmF in strain SEPT362 contributes to adhesion to and invasion of epithelial cells, as well as intra-macrophage survival. In other pathogens, IcmF plays a role in intra-macrophage survival, and adherence to epithelial cells as well (Das et al., 2002). Given that in V. cholerae, IcmF was also implicated in motility (Das et al., 2002), we investigated whether the ΔicmF strain had altered motility. Motility assays showed that the $\Delta icmF$ strain was non-motile, and that motility was restored upon complementation (Fig. 3). Because IcmF is not encoded within the flagella regulon, which is essential for flagella production and motility, these results were initially puzzling. In order to assess the reasons underlying the non-motile phenotype of the $\Delta icmF$ mutant, we investigated expression of several genes from the flagella regulon in this mutant. qRT-PCR assays revealed that flhC, flhD, flgM and fliA flagellar genes were at least 10-fold decreased in $\Delta icmF$, compared to wild-type SEPT362 (Fig. 4). The expression of the ompR (outermembrane protein R) and crp (cAMP receptor protein) genes, which are global regulators of flagellar expression was also measured. While the expression of ompR was not

significantly changed, the expression of *crp* was slightly but significantly decreased in the *icmF* mutant (Fig. 4). These data suggest that an *icmF* mutation is detrimental for the



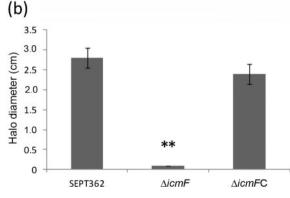


Fig. 3. IcmF is involved in motility. (a) Motility assay on LB 0.3% agar plates after 12h. (b) Motility assay halo measurement after 12 h. **, P<0.05.

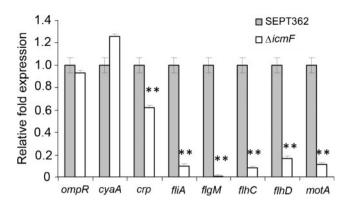


Fig. 4. Relative fold expression of regulators and structural flagellar genes. Statistical significance was determined by Student's t test in comparison with expression in strain SEPT362. **, P<0.05.

expression of the flagella regulon, consequently leading to the loss of motility observed in this mutant.

Role of the IcmF in APEC virulence in chicks

Because the icmF mutant showed decreased adherence to and invasion of epithelial cells, decreased intra-macrophage survival and decreased motility, the contribution of IcmF to virulence in vivo was investigated using a chick infection model. Groups of 16 chicks for each strain were infected with wild-type SEPT362, the icmF mutant and the complemented strain. Chicks infected with the wild-type strain had a 43.75% survival rate on the first day postinfection, and only 12.5% chicks survived on the second day up until the end of the experiment on day 7. Chicks infected with the ΔicmF strain had a 75 % survival rate in the first day post-infection, 37.5 % on the second and third days, and 18.75 % survived until the end of the experiment (Fig. 5). No mortality was observed for the negative PBS control (data not shown). Despite the decreased and delayed mortality in chicks infected with the $\Delta icmF$ mutant, the difference in virulence was not statistically significant. The complementation of the icmF gene in trans partially rescued these phenotypes, but the complemented strain was not as virulent as the wild-type. Because the complementation was performed with the icmF gene cloned into a plasmid, the different infection kinetics of the complemented strain compared to wild-type could be due to differential gene dosage, as well as plasmid maintenance during infection.

DISCUSSION

APEC is responsible for large financial losses in the poultry industry worldwide (Barnes *et al.*, 2003; Gross, 1994; Gyles, 1993), and is also regarded as a potential reservoir for virulence genes for other ExPECs (Johnson *et al.*, 2008; Zhao *et al.*, 2009). Although this *E. coli* pathovar is clearly a burden for food and health safety, its virulence repertoire

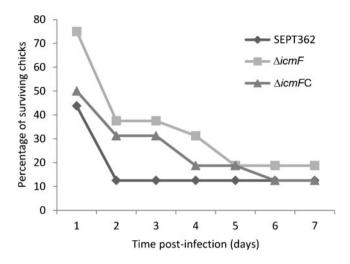


Fig. 5. Virulence test in 1 day-old male chicks. Percentage survival of chicks infected with 10^9 c.f.u. ml^{-1} of SEPT362, $\Delta icmF$ or $\Delta icmF$ complemented strain ($\Delta icmF$ C). Statistical significance was determined by chi-squared test, based on comparison with the SEPT362 strain. Although virulence of the $\Delta icmF$ strain was delayed and diminished, the decrease in virulence was not statistically significant.

remains largely unknown (Dho-Moulin & Fairbrother, 1999; Mellata et al., 2003; Yaguchi et al., 2007; Zhao et al., 2009). Among its known virulence genes, two T6SS genes, clpV and hcp, play an important role in the virulence of APEC strain SEPT362 (de Pace et al., 2010). Here, we describe that another T6SS component, IcmF, is also involved in SEPT362 virulence. IcmF is an innermembrane protein of the T6SS, and is involved in intracellular multiplication inside host cells in other pathogens (Hilbi et al., 2001; Watarai et al., 2001). IcmF is required for survival and replication in macrophages, intracellular growth in amoebae, intracellular replication in eukaryotic cells, and immediate cytotoxicity and exit from the phagosome (Kirby et al., 1998; Pratt & Kolter, 1998; Purcell & Shuman, 1998; VanRheenen et al., 2004).

Here we show that the SEPT362 *icmF* gene is linked to a variety of phenotypes such as adherence, invasion of epithelial cells, survival and replication within macrophages, biofilm formation and motility. The observation that the SEPT362 *icmF* mutant had a decreased intramacrophage survival is congruent with the role of IcmF in other pathogens (Kirby *et al.*, 1998; Pratt & Kolter, 1998; Purcell & Shuman, 1998; VanRheenen *et al.*, 2004).

Fimbriae-mediated adherence is an initial step in colonization, which is important for pathogenesis (Boudeau *et al.*, 2001; Connell *et al.*, 1996; Khan *et al.*, 2007; Marc *et al.*, 1998; Martinez *et al.*, 2000; Mulvey, 2002; Mysorekar & Hultgren, 2006). Here, we report that $\Delta icmF$ shows decreased adherence to HeLa cells *in vitro* when compared to the wild-type strain (Fig. 1). We have previously reported that two T6SS mutants ($\Delta clpV$ and Δhcp) showed

decreased expression of type 1 fimbriae, which led us to speculate that the expression of the T6SS and type 1 fimbriae is coordinated in SEPT362 (de Pace *et al.*, 2010). However, in contrast with the decreased expression of type 1 fimbriae in the *clpV* and *hcp* mutants, expression of these fimbriae was unchanged in the *icmF* mutant, indicating that its adherence defect is not linked to type 1 fimbriae expression.

In addition to adhering to epithelial cells, SEPT362 also adheres to polystyrene, resembling a biofilm. Indeed, the icmF mutant showed a greater decrease in adherence to the plastic surface than it did to the epithelial cells per se, indicating that this mutant could be defective for biofilm formation. Using the crystal violet staining biofilm assay, we showed that the $\Delta icmF$ strain had decreased biofilm formation on polystyrene and glass (Fig. 2). Type 1 fimbriae have been extensively linked to biofilm formation because they provide stable interactions between bacteria and different abiotic surfaces (Marc et al., 1998; Pratt & Kolter, 1998; Schembri & Klemm, 2001). Additionally, Pratt & Kolter (1998) reported that motility is important both for the initial interaction with the surface, and for movement along the surface to form biofilms. Here we show that although the $\Delta icmF$ strain is not defective for type 1 fimbriae expression, it is impaired for motility (Fig. 3). Since motility is involved in biofilm establishment, we suggest that the motility defect in the $\Delta icmF$ strain could be a factor contributing to the defective biofilm formation.

Motility tests showed that the SEPT362 ΔicmF was nonmotile, which corroborates a previous report that an icmF V. cholerae mutant exhibited reduced motility (Das et al., 2002). This result is in contrast with other mutants in the T6SS, namely *clpV* and *hcp*, which have no motility defects (data not shown), suggesting that the motility defect of the icmF mutant is not due to a general defect of motility because of a non-functional T6SS. E. coli strains usually synthesize flagella, which facilitate propulsion and chemotaxis (Apel & Surette, 2008), and are suggested to be involved in mucosal colonization (Ottemann & Miller, 1997). Expression of flagellar genes is hierarchical and coupled with filament formation. Three classes of flagellar genes have been described, class I (early), class II (middle) and class III (late) (Chilcott & Hughes, 2000). The flhD and flhC genes encode transcription factors that form a complex that binds to the upstream regions of fliA, and is a transcriptional activator required for the transcription of the class II operons (Liu & Matsumura, 1994), including flgM, which encodes an anti-sigma factor that interacts with FliA to prevent its association with RNA polymerase for transcription of the class III genes that are FliAdependent, including flagellin itself (fliC) and the motility genes motAB (Liu & Matsumura, 1994). Here we show that transcription of all three flagella gene classes is decreased at least 10-fold (Fig. 4) in the icmF mutant, explaining its impairment in motility. Additionally, we showed that expression of ompR (one of the flagella global regulators) was not altered in the $\Delta icmF$ strain. However, the

expression of *crp*, another global regulator involved in flagellar regulation, is significantly downregulated in the *icmF* mutant, suggesting that some of the effects of IcmF on flagellar expression involve CRP. Considering these results and since IcmF was previously shown to be important in motility (Das *et al.*, 2002), we suggest that this protein is somehow involved in flagellar regulation.

Here we show that IcmF is involved in the pathogenesis of APEC, and that different components of the APEC T6SS make differential contributions to its pathogenesis (de Pace *et al.*, 2010). The observation that the T6SS is also involved in biofilm formation by APEC suggests that this secretion system may play a role in the spread of this pathogen by promoting biofilm formation.

ACKNOWLEDGEMENTS

This work was supported by NIH grant AI053067, grants from FAPESP (grants 2008/56739-6 and 07/50432-3), and grants from CAPES (grant 4062086) and CAPES/PNPD 041/2009. The contents of this work are the sole responsibility of the authors, and do not represent the official views of the NIH NIAID.

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Edited by: M. Whiteley