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The IrgA Homologue Adhesin Iha Is an *Escherichia coli* Virulence Factor in Murine Urinary Tract Infection

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The role of the *Escherichia coli* iron-regulated gene homologue adhesin (Iha) in the pathogenesis of urinary tract infections (UTIs) is unknown. We performed a series of complementary analyses to confirm or refute the hypothesis that Iha is a virulence factor in uropathogenic *E. coli*. Fecal *E. coli* isolates exhibited significantly lower prevalences of *iha* (range, 14 to 22%) than did clinical isolates from cases of pediatric cystitis or pyelonephritis, adult pyelonephritis or urosepsis, or bacteremia (range, 38 to 74%). Recombinant Iha from *E. coli* pyelonephritis isolate CFT073 conferred upon nonadherent *E. coli* ORN172 the ability to adhere to cultured T-24 human uroepithelial cells. In a well-established mouse model of ascending UTI, CFT073 and its derivative UPEC76 (a *pap* [P fimbriae] mutant version of strain CFT073) each significantly outcompeted their respective *iha* deletion mutants in CBA/J mice 48 h after bladder challenge ($P < 0.03$ for urine, both kidneys, and bladders of both constructs, except for bladders of mice challenged with UPEC76 and its deletion mutant, where $P = 0.11$). These data suggest that Iha_{CFT073} is a virulence factor and might be a target for anti-UTI interventions.

Extraintestinal pathogenic *Escherichia coli* cells produce diverse factors (6, 13, 16) that allow them to overcome or subvert host defenses and to colonize, injure, and invade host cells or tissues. Delineation of the roles of these factors could lead to measures to prevent or attenuate infections caused by the organisms that express them (28, 29).

iha encodes the IrgA homologue adhesin (Iha), an outer membrane protein (OMP) first characterized in *E. coli* O157:H7, which confers adherence to nonadherent laboratory strains (41). *iha* occurs frequently among uropathogenic *E. coli* strains (3, 16–18, 22, 23, 25, 27). Its designation is derived from the similarity of Iha to IrgA (9), which is postulated to play a role in the colonization of mice experimentally infected with *Vibrio cholerae* (10).

$iha_{O157:H7}$ and open reading frame R4 in pathogenicity-associated island 1 (PAI-1) of the well-studied *E. coli* pyelonephritogenic strain CFT073 (serotype O6:K2:H1) (12) differ by five synonymous nucleotide changes and one nonsynonymous nucleotide change. Strain UPEC76, a derivative of CFT073 in which both pyelonephritis-associated *pap* (pilus associated with pyelonephritis; P fimbriae) operons, in separate PAIs, have been inactivated by deletions, colonizes the urinary tract in a mouse model of ascending urinary tract infection (UTI) as well as does its parent (32). This finding suggests that CFT073 requires non-*pap* loci for virulence, with *iha* being one such candidate critical locus. Also, the virulence of a *Proteus mirabilis* strain, in which *irgA* was identified by signature-tagged mutagenesis, was attenuated in a mouse model of ascending UTI (4).

Because Iha's status as a virulence factor has been incompletely elucidated, we performed complementary studies to test the hypotheses that iha^+ extraintestinal pathogenic *E. coli* *iha* is epidemiologically associated with human disease, that cloned *iha* confers the ability to adhere to uroepithelial cells, and that Iha_{CFT073} is needed for complete urovirulence in mice challenged with strain CFT073 or UPEC76.

MATERIALS AND METHODS

Bacteria and plasmids used. *E. coli* strains CFT073, UPEC76 (32), and 86-24 (serotype O157:H7) (41) have been previously described. Ampicillin and nalidixic acid concentrations in media were 200 and 20 μg ml^{-1}, respectively. Cloned PCR products were transformed into *E. coli* strains DH5α (36) and ORN172, a laboratory *E. coli* strain devoid of all known adherence mechanisms including type 1 pili (46). Nalidixic acid-resistant CFT073 ($CFT073^{nalR}$) was derived by plating ca. 10^{10} CFT073 cells on Luria-Bertani (LB) agar containing nalidixic acid and selecting a spontaneously resistant mutant. *E. coli* SM10(λpir) (38) was transformed with, and then was the donor for, suicide plasmid constructs. *E. coli* strain B171 (O111:NM) (33) was used as a positive adherence control. $pSK+_{Iha\text{-}O157:H7}$, formerly pIha, is pSK+ (Stratagene, La Jolla, Calif.) containing cloned $iha_{O157:H7}$ (41). To clone and express Iha_{CFT073}, we amplified CFT073 DNA with primers A (5′GGGGATCCA ATTCTGGCATGCCGAGGCAGTGC3′) and B (5′GGTCTAGATTCTCGTTG CCACTGTTCCGCCAGG3′) (41), containing engineered 5′ BamHI and XbaI sites, respectively. This amplicon, consisting of iha_{CFT073} and 141 and 80 bp 5′ and 3′ to its termini, respectively, was digested with BamHI and XbaI and cloned into the corresponding sites in pSK+, resulting in $pSK+_{Iha\text{-}CFT073}$. It was then sequenced bidirectionally.

Deletion of iha_{CFT073} from CFT073 and UPEC76. To create an in-frame, unmarked, isogenic deletion of *iha* in strains CFT073 and UPEC76, we cloned an in-frame deletion construct of the target gene into suicide plasmid pCVD442 (5). We used inverse PCR (42) to obtain a candidate DNA sequence (not shown) 5′ to iha_{CFT073}. We then used primer C (5′GCAGAGCTCCCTTGCAAGAGGG CGTCGAGC3′) and 5′GCTATGGATCCGGCTGAAAATCCGAGACAGGG3′ to produce a 704-bp amplicon that includes the 5′ terminus of iha_{CFT073} and 630 bp

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of the 5′ noncoding region. This amplicon was sequentially cloned into, and excised from, the pGem-TEasy vector and pSK+ with SacI and BamHI. Next, the cloned 558-bp amplicon that spans the $iha_{O157:H7}$ 3′ terminus (41), which differs from the corresponding region in CFT073 by only 3 nucleotides in the downstream noncoding region of the gene, was excised from pSK+ with BamHI and XhoI and ligated to the SacI-BamHI fragment spanning the 5′ iha_{CFT073} terminus. This fusion, representing a central truncation of iha_{CFT073}, was then cloned into and excised from the pSK^+ SacI and XhoI sites and finally inserted into the SacI and XhoI sites of pCVD442 (5), producing pCVD442Δiha_{CFT073}. Though the 5′ end of primer C imperfectly represents the *iha* upstream region, the deletion mutant is, as intended, identical to its parent at that site (sequence data not shown).

$CFT073^{nalR}$ or UPEC76 was mated separately with *E. coli* SM10(λpir) transformed with pCVD442Δiha_{CFT073} on LB agar at 37°C. Transconjugants were selected by plating mated bacteria on LB agar containing ampicillin and nalidixic acid. The resulting presumed merodiploids were grown (37°C in LB broth [36]), plated onto LB agar containing 5% sucrose but no NaCl, and incubated (30°C). Sucrose-resistant, ampicillin-susceptible colonies from these matings were designated CFT073Δiha_{CFT073} and UPEC76Δiha_{CFT073}, respectively.

Deletion mutant characterizations. Genomic DNA from the presumptive mutants and their parents was digested with BstXI, electrophoresed, transferred to a MagnaCharge nylon membrane (GE Osmonics, Minnetonka, Minn.), and probed with $iha_{O157:H7}$. A 371-bp amplicon produced by primers A and B from each candidate deletion mutant was cloned into pSK+ and sequenced. The deletion mutants were serotyped by Flemming Scheutz, digested with XbaI, and analyzed by pulsed-field gel electrophoresis, and tested by PCR for 11 (*iha*, *papA*, *papC*, *sfa*/*focDE*, *focG*, *fimH*, *iutA*, *fyuA*, *iroN*, *hlyD*, and *malX*) (11, 26) putative or demonstrated urovirulence loci that are known to be present in the parent plus, specifically, the F7-1 and F7-2 *papA* alleles (21, 22). The deletion mutants and their parents were also evaluated for mannose-sensitive agglutination of guinea pig erythrocytes and baker's yeast and for mannose-resistant agglutination of human erythrocytes after overnight growth in static broth or on agar plates to indicate expression of type 1 or P fimbriae, respectively (24), and for differences in growth rates (compared to parents) over 18 h in LB broth shaken at 37°C. Finally, the mutants were hybridized with digoxigenin-labeled pCVD442 to confirm suicide plasmid loss.

Anti-Iha antibodies. The PolyQuik protocol (Zymed Laboratories, South San Francisco, Calif.) and affinity purification produced lapine polyclonal antibodies against an Iha epitope. The resulting affinity-purified antibodies (designated anti-Iha antibodies) do not detect an externally directed Iha epitope (41).

OMP analyses. OMPs were prepared (1) from bacteria grown overnight at 37°C in 100 ml of LB broth or Dulbecco's minimal essential medium (D-5030; Sigma, St. Louis, Mo.) containing 0.45% glycerol, 25 mM HEPES, 10 mM sodium pyruvate, 4.5 mN sodium hydroxide, 4 mM L-glutamine, and 44 mM sodium bicarbonate (DMEM). Protein concentrations were determined (protein assay kit; Bio-Rad, Hercules, Calif.). OMPs (2 μg) in loading buffer were electrophoretically separated (sodium dodecyl sulfate–10% polyacrylamide) and transferred to membranes (Immobilon-P; Millipore, Billerica, Maine) or Coomassie stained to confirm equal loading of analyte. The membranes to which the proteins were transferred were blocked overnight in antibody buffer (phosphate-buffered saline with 0.05% [vol/vol] Tween 20) containing 5% nonfat dried milk and 0.02% sodium azide) and washed once with antibody buffer. The membranes were then incubated overnight with anti-Iha antibodies diluted 1:2,000 in antibody buffer, washed three times with antibody buffer, incubated for 30 min with affinity-purified goat anti-rabbit immunoglobulin G (heavy plus light chains) peroxidase conjugate (Boehringer Mannheim, Indianapolis, Ind.) diluted 1:2,000 in antibody buffer, and washed again three times with antibody buffer. Membranes were incubated at 4°C or room temperature (blocking or all subsequent steps, respectively). Bound antibodies were detected with the use of the SuperSignal Western blotting (Pierce, Rockford, Ill.).

Adherence assay. T-24 human bladder epithelial cells (30), after growth to confluence at 37°C in 5% CO_2 in plastic flasks in culture medium (McCoy's 5a medium; Sigma; M-4892) supplemented with 10% (vol/vol) heat-inactivated fetal bovine serum, were trypsinized, pelleted, resuspended in the above medium, and then added to wells (in 2-ml volumes) of a six-well plastic flat-bottom cell culture plate (Costar 3516; Corning, Corning, N.Y.) containing sterile plastic 25-mm-diameter coverslips. When cells were ca. 75% confluent, the medium was replaced with fresh medium containing mannose (0.5%) and ampicillin, and adherence assays were performed.

E. coli strain B171 and *E. coli* ORN172 containing $pSK+_{Iha\text{-}CFT073}$ or pSK+ were grown in LB broth with ampicillin at 37°C without shaking, before assessment in the adherence assay. Cells and bacteria were incubated for 1 (wild type and wild-type derivatives) or 2 h (ORN172 transformants) at 37°C in 5% CO_2, after which they were washed eight times with sterile phosphate-buffered saline, fixed (100% methanol, 1 min), stained (0.5% crystal violet, 15 min), and thoroughly washed with water to remove background stain. Coverslips were then air dried and mounted on glass slides, and adherent organisms were enumerated by a microscopist unaware of the identity of the organisms in each well. The test organisms were concurrently assessed in duplicate on each of multiple days. Five fields were randomly selected for analysis from each of two duplicate wells per test organism. The adherent organisms were counted separately for each cell within each selected field.

Molecular epidemiological analyses. *iha* was sought in *E. coli* from the feces of healthy volunteers and noninfected hospitalized subjects and from *E. coli* recovered from the urine, blood, or cerebrospinal fluid of infected patients by PCR with internal primers D (5′CTGGCGGAGGCTCTGAGATCA3′) and E (5′TC CTTAAGCTCCCGCGGCTGA3′) and/or by dot blot hybridization with an internal digoxigenin-labeled *iha* probe (synthesized with primers D and E) (22). Isolates were tested in duplicate, with independently prepared boiled lysates, for the presence of *iha*. Strains CFT073 and K-12 were the positive and negative controls, respectively (22, 25).

Mouse models of UTI. In an extensively evaluated ascending atraumatic model of UTI, 6- to 10-week-old CBA/J mice were anesthetized and inoculated via the urethra with 1.0 μl of the test bacterial suspension/g of body $weight^{-1}$, under conditions that avoided vesicoureteral reflux (14, 15, 35). Challenge bacteria were grown in shaking, overnight LB broth cultures at 37°C, pelleted, and resuspended in LB broth before challenge. After a standard 2-day period, mice were euthanized, and aseptically harvested urine and bladder and kidney homogenates were cultured quantitatively on agar. Dual-strain challenges were used to compare the colonizing abilities of CFT073 and of UPEC76 to those of their respective *iha* deletion mutants. Intra-animal competition assessments were used in these studies because they minimize the impact of mouse-to-mouse variation and maximize the ability to identify differences among test strains. The challenge inocula contained ca. 2×10^9 total CFU, which is standard in our use of this model and which is within the range used by others (7, 32, 43) in various mouse models of UTI.

Because we used unmarked mutations in competition experiments, neither CFT073 nor UPEC76 could be easily phenotypically differentiated from their respective iha_{CFT073} mutants in the postmortem cultures in mouse UTI models. Therefore, we tested colonies from each mouse culture, randomly selected to preclude systematic bias favoring one test strain over the other, by using dot blot hybridization with an internal *iha* probe or PCR with internal *iha* primers D and E (25) to determine the relative proportions of recovered organisms that contained an intact copy of *iha*. Each isolate was analyzed in duplicate, with the corresponding challenge strains used as controls. Colonies from the inoculum suspension cultures were similarly analyzed to define the relative abundances of the two test strains as administered to the mice, and this proportion (the input ratio) was used to adjust the postmortem quantitative culture results (the output ratio) from the mouse infection experiments to obtain the competitive index (CI). Between 10 and 64 colonies per culture (i.e., from inoculum suspensions and postmortem samples) were so assessed. In addition, to exclude contamination, one putative representative of each test strain from each positive culture from the mouse experiments was compared with the actual test strain by random amplified polymorphic DNA analysis (22, 44).

Statistics. Differences in proportions were tested for significance by Fisher's exact test for unpaired comparisons or McNemar's test for paired comparisons. Differences involving continuous variables were assessed for significance by the Mann-Whitney U test and the Wilcoxon rank sum tests for unpaired and paired comparisons, respectively. The threshold for statistical significance was a P value <0.05, but selected values <0.10 are noted in Table 2 to demonstrate trends.

Nucleotide sequence accession number. The nucleotide sequence of the region upstream of iha_{CFT073} in CFT073 has been deposited in the GenBank database under accession number AF401752.

RESULTS

Molecular epidemiology of *iha*. To determine the epidemiological association of *iha* with specific extraintestinal infection syndromes, *iha* was sought among 286 fecal *E. coli* isolates and 839 clinical isolates from diverse human populations (Table 1). Fecal isolates consistently exhibited a lower prevalence of *iha* (range, 14 to 22%) than did clinical isolates from all infection syndromes except cystitis in women and neonatal meningitis. The highest prevalences of *iha* (50 to 74%) occurred among

TABLE 1. Prevalence of *iha* among fecal and clinical isolates of *E. coli* from diverse host populations

Group no.	Host population (sample source) and/or syndrome	*iha* proportion (%)	*P*: (by Fisher's exact test) for comparison with group no.:													Reference[b] for:	
			1	2	3	4	5	6	7	8	9	10	11	12	13	Isolates	*iha* data
1	Healthy women (fecal)	13/77 (17)															
2	Healthy children (fecal)	9/46 (20)	—														
3	Healthy adults (fecal)	20/92 (22)	—	—													
4	Hospitalized veterans (fecal)	10/71 (14)	—	—	—											36a	36a
5	Pediatric cystitis	26/39 (74)	***	***	***	***										19	
6	Pediatric cystitis	22/44 (50)	***	**	***	***	—									2	
7	Pediatric pyelonephritis	25/48 (52)	***	***	***	***	—	—								2	
8	Women, cystitis	12/74 (16)	—	—	—	—	***	***	***							22	22
9	Women, cystitis	22/82 (27)	—	—	—	—	***	***	***	—							
10	Women, pyelonephritis	97/170 (57)	***	***	***	***	—	—	—	***	***					42	
11	Adults, urosepsis	37/66 (56)	***	***	***	***	—	—	—	***	***	—				26	26
12	Adults, bacteremia	69/182 (38)	***	*	**	***	***	—	—	***	—	***	**			36a	36a
13	Veterans, bacteremia	25/64 (39)	***	*	*	**	**	—	—	**	—	*	—	—			
14	Neonatal meningitis	13/70 (19)	—	—	—	—	***	***	***	—	—	***	***	**	**	24a	24a

[a] *P* value symbols: —, $P > 0.05$; *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$.
[b] If sources for these collections were not this study.

cystitis and pyelonephritis isolates from children and pyelonephritis and urosepsis isolates from adults. Intermediate prevalences (38 to 39%) that still significantly exceeded control frequencies were observed among bacteremia isolates from diverse sources. These differences demonstrate that Iha fulfills the first of the molecular restatement of Koch's postulates, which requires that the property of interest be epidemiologically associated with disease (8).

Structure of iha_{CFT073} and its upstream region. Iha_{CFT073} has a deduced molecular size of 78 kDa and contains 696 amino acids. Iha_{CFT073} and $Iha_{O157:H7}$ differ by only one amino acid (an $Asp^{469} \rightarrow Asn^{469}$ alteration), but the two *iha* genes differ by 6 nucleotides. Upstream of the *iha* ATG start codon, CFT073 and *E. coli* O157:H7 are 98.8 and 34.8% identical from position −1 to −489 and positions −490 through −630, respectively, consistent with CFT073's extensive chromosomal mosaicism (45).

Role of Iha_{CFT073} in adherence of *E. coli* to uroepithelial cells. $pSK+_{Iha\text{-}CFT073}$ conferred on *E. coli* ORN172 the ability to adhere to cultured T-24 uroepithelial cells, whereas pSK+ did not (Fig. 1). The adherence pattern observed for *E. coli* ORN172 transformed with $pSK+_{Iha\text{-}CFT073}$ was diffuse, and there was interfield variability. This adherence pattern of Iha recombinants was also observed on HEp-2 cells (data not shown). Strain B171 displayed its characteristic localized adherence to cells (data not shown). Assessments of the cellular adherence of CFT073 or UPEC76, their *iha* deletion mutants, or their deletion mutants transformed with $pSK+_{Iha\text{-}CFT073}$ and pSK+ were precluded by the considerable cytotoxicity of these strains (31), even after only 1 h of incubation (data not shown).

Comparisons of CFT073 to CFT073Δiha_{CFT073}, and of UPEC76 to UPEC76Δiha_{CFT073}. Compared with their respective parents, CFT073Δiha_{CFT073} and UPEC76Δiha_{CFT073} exhibit the same growth characteristics, colony morphology, O:K:H serotype, XbaI pulsed-field gel electrophoresis profile, baker's yeast and erythrocyte agglutination patterns, and extended virulence genotype (except, of course, for iha_{CFT073}).

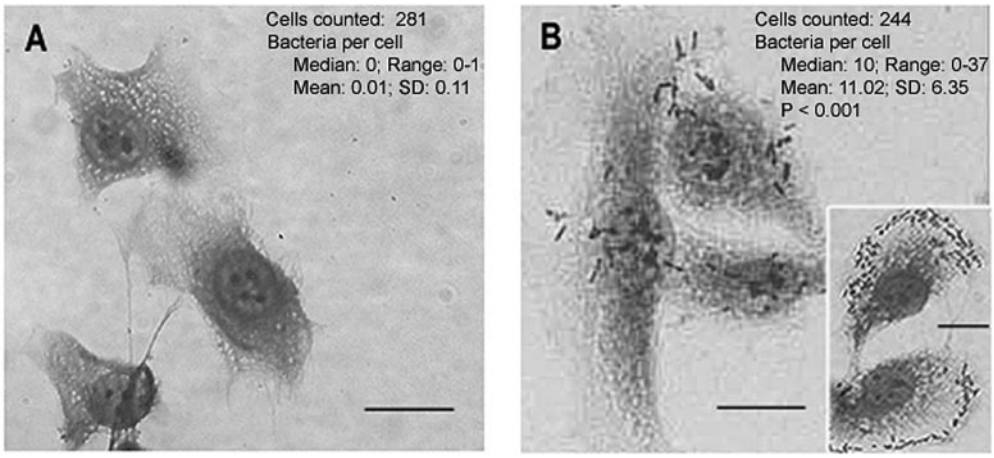

FIG. 1. Adherence of laboratory *E. coli* to T-84 uroepithelial cells, with and without cloned Iha. Shown is *E. coli* ORN172 transformed with pSK+ (A) or $pSK+_{Iha\text{-}CRT073}$ (B). Panel B demonstrates the typical adherence of *E. coli* ORN172 transformed with $pSK+_{Iha\text{-}CFT073}$ to cells, but the adherence intensity is variable, with some cells quite densely covered (B, inset). Numbers are values associated with cellular adherence for the corresponding recombinant strains. The *P* value in panel B is relative to the vector control (Mann-Whitney U test). Bars, 20 μm.

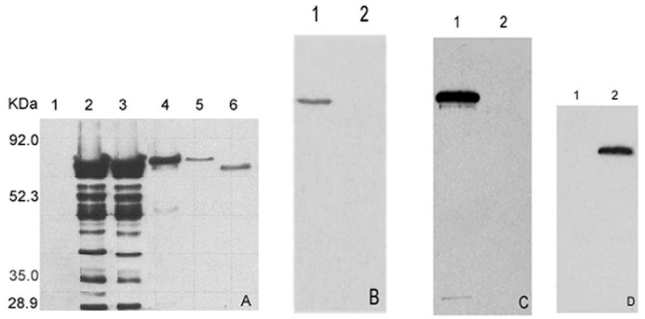

FIG. 2. OMPs of *E. coli* ORN172, with and without recombinant Iha_{CFT073}, and wild-type and derivative *E. coli*. (A) Immunoblots of OMPs from strain ORN172 transformed with pSK+ (lane 1), $pSK+_{Iha\text{-}O157:H7}$ (lane 2), $pSK+_{Iha\text{-}CFT073}$ (lane 3), *E. coli* CFT073 (lane 4), UPEC76 (lane 5), and *E. coli* O157:H7 (lane 6). (B to D) Immunoblots of OMPs from CFT073 (B, lane 1), CFT073Δ$iha_{\text{-}CFT073}$ (B, lane 2), UPEC76 (C, lane 1), UPEC76Δ$iha_{\text{-}CFT073}$ (B, lane 2), UPEC76 (C, lane 1), UPEC76Δ$iha_{\text{-}CFT073}$ (C, lane 2), and CFT073 grown in LB broth (D, lane 2) or DMEM (D, lane 2). The single immunoreactive bands in panels B to D represent the 76-kDa Iha antigen.

By Southern hybridization, 4.7-kb BstXI fragments detected by the *iha* probe in CFT073 and UPEC76 were not evident in their respective Δiha_{CFT073} mutants. Most importantly, sequencing across the target deletion site demonstrated a 1,939-bp deletion in CFT073Δiha_{CFT073} and UPEC76Δiha_{CFT073}, with the intended insertion of GATC. This resulted in the in-frame replacement of the 647 amino acids between Glu^{25} and Gln^{673} with a Gly and a Ser.

OMP analysis. Anti-Iha antibodies detected abundant 76-kDa antigen, as well as probable breakdown products, in OMPs from *E. coli* ORN172 transformed with $pSK+_{Iha\text{-}O157:H7}$ or $pSK+_{Iha\text{-}CFT073}$ (Fig. 2A). OMPs from strains CFT073 and UPEC76 produced less intensely immunoreactive 76-kDa Iha (Fig. 2A). As expected, OMPs from *iha* deletion mutants UPEC76Δiha_{CFT073} and CFT073Δiha_{CFT073} did not exhibit Iha antigen (Fig. 2B and C). Iha was not detectable when CFT073 was grown in LB broth but was detectable when it was grown in DMEM (Fig. 2D).

In vivo challenge experiments. CBA/J mice were challenged via the urethra with mixtures of CFT073 and CFT073Δiha_{CFT073} or mixtures of UPEC76 and UPEC76Δiha_{CFT073}, in dual-strain competition model experiments. Urine, bladders, and kidneys harvested 48 h after inoculation uniformly showed greater overall intensity and prevalence of colonization with CFT073 or UPEC76 than with these strains' respective Δiha_{CFT073} mutants, as summarized for categorical outcomes in Table 2 and as illustrated for absolute $\log_{10}$ CI values in Fig. 3. Of note, many cultures yielded only the parent strain, indicating that the mutants were present at levels below the detection threshold or were lost in vivo. These data establish the contribution of iha_{CFT073} to bladder and kidney colonization in mice challenged with a urovirulent *E. coli* strain, with or without an intact *pap* operon.

DISCUSSION

The impaired abilities to colonize the mouse urinary tract displayed by isogenic, in-frame, iha_{CFT073} deletion mutants in two different *E. coli* backgrounds provide strong experimental support for the hypothesis that Iha is a urovirulence factor. That Iha_{CFT073} conferred the ability to adhere to T-24 cells is particularly interesting, because *E. coli* adherence to these cells of human bladder origin has been reported to be independent of known UTI-associated adhesins, such as type 1, P, or S fimbriae, or afimbrial adhesin I (30). These assay results also demonstrate that the single amino acid difference between Iha_{CFT073} and $Iha_{O157:H7}$ does not negate the ability of the cloned protein to confer cellular adherence capability to nonadherent laboratory *E. coli*. This phenotype, though anticipated, warranted confirmation because of the known ability of single amino acid polymorphisms to alter the receptor binding characteristics of the pathogenetically important *E. coli* adhesin, FimH (39). Whether Iha augments adherence directly or indirectly via effects on other bacterial components and, if it acts directly, whether this phenotype involves receptor-specific or nonspecific binding to the host cell await further assessment.

TABLE 2. Comparative urovirulences of CFT073 and UPEC76 versus their respective Δiha_{CFT073} derivatives in the mouse model of ascending UTI (competition experiments)

Parent	Site cultured	Total no.[c]	No. (%)[a] with:		P^d	No. (%)[b] with:		P^d
			Parent > Δiha_{CFT073} mutant	Δiha_{CFT073} mutant > parent		Parent only	Δiha_{CFT073} mutant only	
$CFT073^{nalR}$	Urine	19	12 (63)	2 (11)	<0.02	8 (42)	0 (0)	<0.01
	Bladder	19	16 (84)	3 (16)	<0.01	2 (11)	0 (0)	
	Right kidney	19	11 (58)	1 (5)	<0.01	2 (11)	0 (0)	
	Left kidney	19	11 (58)	1 (5)	<0.01	4 (21)	0 (0)	
UPEC76	Urine	24[c]	17 (71)	5 (21)	0.017	7 (29)	1 (4)	0.07
	Bladder	30	18 (60)	11 (37)		6 (20)	0 (0)	0.03
	Right kidney	30	19 (63)	7 (23)	0.03	8 (27)	3 (10)	
	Left kidney	30	16 (53)	8 (27)		9 (30)	5 (17)	

[a] Percentages do not sum to 100% in all instances because some cultures (included in denominators) yielded no growth. Comparative prevalence of the competing strains was assessed based on the CI, i.e., after adjusting the output ratio from that culture for the input ratio from the same experiment.
[b] Percentages do not sum to 100% because some cultures yielded no growth or growth of both competing strains.
[c] Urine was unavailable for six mice from the UPEC76 experiments.
[d] *P* values (by McNemar's test; two tailed) are provided where $P < 0.10$.

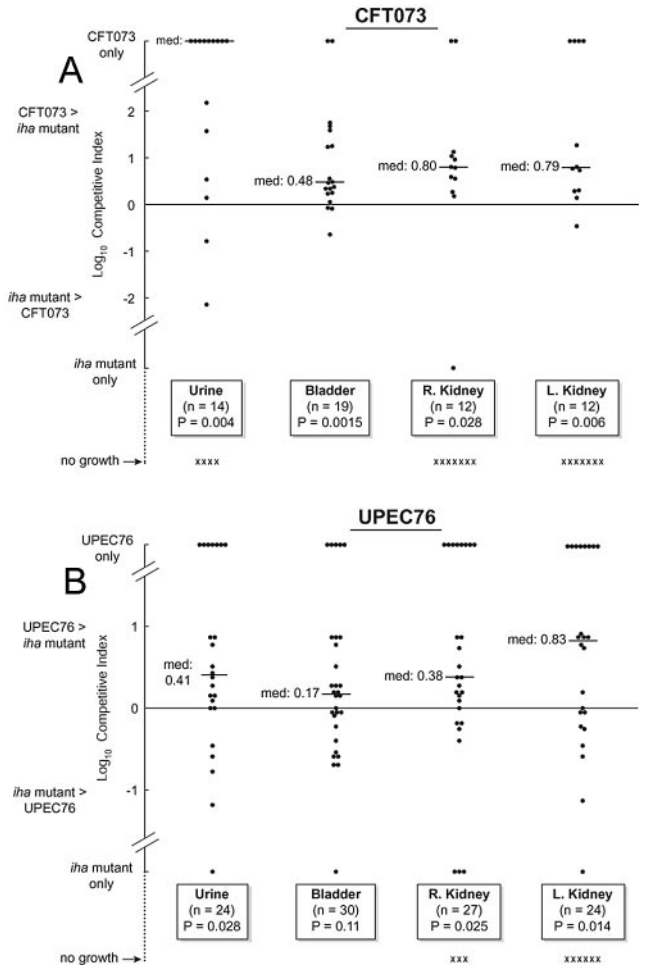

FIG. 3. Comparative urovirulences in mice of CFT073 and UPEC76 versus those of their respective Δ*iha*$_{CFT073}$ mutants. Mice were challenged via the urethra with 20 μl containing ca. 2×10^9 CFU of a mixture of CFT073 and CFT073Δ*iha*$_{CFT073}$ (A) or UPEC76 and UPECΔ*iha*$_{CFT073}$ (B). CIs were calculated as the parent/deletion mutant ratios, normalized to the corresponding input ratio. X, no growth; horizontal bars, median values.

Additionally, our work confirms the localization of Iha to the cell envelope of another *iha*$^+$ strain and demonstrates that its presence in the cell envelope of CFT073, like the presence of the Iha homologue in the cell envelope of locus of enterocyte effacement-negative *E. coli* O91:H$^-$ strain 4797/97 (37), depends on the growth medium of the organism, with expression being suppressed in LB broth. Indeed, this diminished expression in LB broth might have actually led to an underattribution of the role of Iha in the mouse colonization experiments, because the challenge inocula were prepared in LB broth, which would tend to diminish the expression of Iha in the parent at the time of inoculation.

The mutations that produced the colonization deficiencies of CFT073 and UPEC76 were each in-frame deletions of *iha*$_{CFT073}$. Such deletions are quite unlikely to exert polar effects on downstream genes, although we cannot exclude completely the possibility that occult *cis*-acting effects of the deletion reduced colonization ability. Additionally, the deletion mutants were indistinguishable from the parent with respect to a broad panel of characteristics analyzed other than *iha*$_{CFT073}$ and antigenic Iha$_{CFT073}$. Moreover, two independent deletions of *iha*$_{CFT073}$ in two different organisms produced the same diminished-colonization phenotype, to similar extents, without perturbing other analyzed loci. Recent data demonstrate the fairly frequent occurrence of secondary mutations in extraintestinal pathogenic *E. coli* using pCVD442 (20); therefore, it is important to generate such confirmatory results to increase confidence that the altered phenotype actually was caused by the intended mutation. The extensive methods that we used to exclude unanticipated secondary mutations in the *iha* mutants exceed those customarily used for this purpose in studies of this sort and were the same as those that uncovered otherwise-occult secondary mutations in the recent study; their use increases confidence that such alterations were absent from the present mutants.

Although inactivating *papG* in *E. coli* pyelonephritis strain DS17 (O6:K5) by introducing a premature stop codon reduced the ability of this strain to cause ascending pyelonephritis in monkeys (34), inactivation of both *pap* operons in CFT073 did not diminish CFT073's ability to colonize the mouse urinary tract (32). Likewise, inactivating the putative virulence factor *sat* (secreted autotransporter toxin) in CFT073 had no impact on in vivo colonization ability (32). Thus, *iha* joins *tonB*, *iutA*, *chuA*, and *fimH* as the only known putative urovirulence genes, the mutation of which in the CFT073 background significantly attenuates urovirulence (43). Moreover, *iha*$_{CFT073}$ is the first such gene for which an unmarked, in-frame deletion was used to generate the evaluated CFT073 mutant.

Finally, our epidemiological data provide novel molecular evidence that *iha* is statistically significantly more frequent among diverse groups of extraintestinal *E. coli* pathogens than among fecal control isolates. The absence of a categorical association of *iha* with all infection syndromes and host groups suggests differing pathogenetic mechanisms across clinical and epidemiological settings, a worthy topic for future investigations.

In summary, Iha$_{CFT073}$'s demonstrated pathogenetic importance in a mouse model of UTI complements (i) epidemiological data associating *iha* with recurrent or invasive UTI and diverse-source bacteremia and (ii) in vitro uroepithelial adherence data. However, even though *iha*$_{CFT073}$ mutants exhibited impaired in vivo colonization in two different bacterial hosts and although this colonization deficit is plausibly attributable to *iha*'s adherence-conferring function, Iha $_{CFT073}$ has not yet been proven to serve as an adhesin in wild-type pathogens. Nonetheless, our data suggest that Iha deserves further scrutiny as a molecule to exploit for prevention or treatment of human UTIs.

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