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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 IhaCFT073 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).

ihaO157: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 (pili 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 iagA 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 IhaCFT073 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 20 and 20 μg/ml, 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. 1010 CFT073 cells on Luria-Bertani (LB) agar containing nalidixic acid and selecting a spontaneously resistant mutant. E. coli SM10(pΔpir) (38) was transformed with, and then was the donor for, suicide plasmid constructs. E. coli strain B711 (O111:NM) (33) was used as a positive adherence control. pSK-rIhaO157:H7, formerly plha, is pSK+ (Strategene, La Jolla, Calif.) containing cloned ihaO157:H7 (41). To clone and express IhaCFT073, we amplified CFT073 DNA with primers A (‘5’GGGAGATCC AATCTGCGATGCAGCGAAGGGGGGGATCCA 3’) and B (‘5’GGGAGATCC AATCTGCGATGCAGCGAAGGGGGGGATCCA 3’) (41), containing engineered 5′ BamHI and XbaI sites, respectively. This amplicon, consisting of ihaCFT073 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+ihaCFT073. It was then sequenced bidirectionally.

Deletion of ihaCFT073 from CFT073 and UPEC76. To create an in-frame, unmarked, inogenic 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 ihaCFT073. We then used primer C (‘5’GGCAGAGCTCCCTGCGAAGGG GGGGGGGATCCA 3’) and 5′GGCAGAGCTCCCTGCGAAGGG GGGGGGGATCCA 3’) to produce a 704-bp amplicon that includes the 5′ terminus of ihaCFT073 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 \( iha \) terminus (3’ terminus) 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’ \( iha \) terminus. This fusion, representing a central truncation of \( iha \), was then cloned into and excised from the pSK− SacI and XhoI sites and finally inserted into the SacI and XhoI sites of pCD424 (5), producing pCD424A::\( iha \). 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 or ORN172 was mated separately with \( E. coli \) SM10Apr (transconjugants) or E. coli CFT073 strains harboring pSK+::\( iha \) or pSK−::\( iha \), after growth to an optical density of 0.5 at 600 nm (OD600). 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]) on plates on LB agar containing 5% sucrose but no NaCl, and incubated (30°C). Sucrose-resistant, ampicillin-susceptible colonies from these matings were designated CFT073A::\( iha \) or CFT073B::\( iha \), respectively.

Deletion mutant characterizations. Genomic DNA from the presumptive mutants and their parents was digested with Bsal, electrophoresed, transferred to a MagnaCharge nylon membrane (GE Osmonics, Minnetonka, Minn.), and probed with \( iha \). 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 Fleming Scheutz, digested with XbaI, and analyzed by pulsed-field gel electrophoresis, and tested for PCR for \( iha \) (data not shown; \( iha \), \( papA \), \( fimH \), \( fyuA \), \( hlyD \), \( inv \\& \textbf{and} E(5’-terminus) \) and/or by dot blot hybridization with an internal digoxigenin-labeled probe (synthesized with primers D and E) (22). Isolates were tested in duplicate, with independently prepared boiled lyses, 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 BALB/c mice were anesthetized and inoculated via the urethra with 1.0 \( \mu \)l 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 colonization abilities of CFT073 and of UPEC76+::\( iha \) in the presence 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 \( \times \) \( 10^7 \) 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 \( iha \) deletion mutants in 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) 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) were similarly analyzed to define the relative abundances of the two test strains. The threshold for statistical significance was a \( P \) value < 0.05, but selected values < 0.10 are noted in Table 2 to demonstrate trends.

**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).

**Download from:** https://iai.asm.org/ on February 8, 2014 by Washington University in St. Louis
amino acids. IhaCFT073 and IhaO157:H7 differ by only one amino acid; the deduced molecular size of 78 kDa and contains 696 cysteines 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\textsubscript{CFT073} and its upstream region.** Iha\textsubscript{CFT073} has a deduced molecular size of 78 kDa and contains 696 amino acids. Iha\textsubscript{CFT073} and Iha\textsubscript{O157:H7} differ by only one amino acid (an Asp\textsubscript{469}→Asn\textsubscript{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\textsubscript{CFT073} in adherence of *E. coli* to uroepithelial cells.** pSK\textsuperscript{+}\textsubscript{Iha-CFT073} conferred on *E. coli* ORN172 the ability to adhere to cultured T-24 uroepithelial cells, whereas pSK\textsuperscript{+} did not (Fig. 1). The adherence pattern observed for *E. coli* ORN172 transformed with pSK\textsuperscript{+}\textsubscript{Iha-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\textsuperscript{+}\textsubscript{Iha-CFT073} and pSK\textsuperscript{+} 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\textsubscript{CFT073}, and of UPEC76 to UPEC76Δiha\textsubscript{CFT073}**. Compared with their respective parents, CFT073Δiha\textsubscript{CFT073} and UPEC76Δiha\textsubscript{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\textsubscript{CFT073}).

<table>
<thead>
<tr>
<th>Group no.</th>
<th>Host population (sample source) and/or syndrome</th>
<th><em>iha</em> proportion (%)</th>
<th>Reference\textsuperscript{a} for:</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Healthy women (fecal)</td>
<td>13/77 (17)</td>
<td>36a 36a</td>
</tr>
<tr>
<td>2</td>
<td>Healthy children (fetal)</td>
<td>9/46 (20)</td>
<td>19</td>
</tr>
<tr>
<td>3</td>
<td>Healthy adults (fetal)</td>
<td>20/92 (22)</td>
<td>2</td>
</tr>
<tr>
<td>4</td>
<td>Hospitalized veterans (fetal)</td>
<td>10/71 (14)</td>
<td>22 22</td>
</tr>
<tr>
<td>5</td>
<td>Pediatric cystitis</td>
<td>26/39 (74)</td>
<td>42</td>
</tr>
<tr>
<td>6</td>
<td>Pediatric cystitis</td>
<td>22/44 (50)</td>
<td>26 26</td>
</tr>
<tr>
<td>7</td>
<td>Pediatric pyelonephritis</td>
<td>25/48 (52)</td>
<td>36a 36a</td>
</tr>
<tr>
<td>8</td>
<td>Women, cystitis</td>
<td>12/74 (16)</td>
<td>11</td>
</tr>
<tr>
<td>9</td>
<td>Women, cystitis</td>
<td>22/92 (27)</td>
<td>6 2</td>
</tr>
<tr>
<td>10</td>
<td>Women, pyelonephritis</td>
<td>97/170 (57)</td>
<td>12</td>
</tr>
<tr>
<td>11</td>
<td>Adults, urosepsis</td>
<td>37/66 (56)</td>
<td>13</td>
</tr>
<tr>
<td>12</td>
<td>Adults, bacteremia</td>
<td>69/182 (38)</td>
<td>14</td>
</tr>
<tr>
<td>13</td>
<td>Veterans, bacteremia</td>
<td>25/64 (39)</td>
<td>15</td>
</tr>
<tr>
<td>14</td>
<td>Neonatal meningitis</td>
<td>13/70 (19)</td>
<td>16</td>
</tr>
</tbody>
</table>

\textsuperscript{a} P value symbols: —, P > 0.05; *, P ≤ 0.05; **, P ≤ 0.01; ***, P ≤ 0.001.

\textsuperscript{b} If sources for these collections were not this study.

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\textsuperscript{+} (A) or pSK\textsuperscript{+}\textsubscript{Iha-CFT073} (B). Panel B demonstrates the typical adherence of *E. coli* ORN172 transformed with pSK\textsuperscript{+}\textsubscript{Iha-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-CFT073 (lane 2), pSK+Iha-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), CFT073Iha-CFT073 (B, lane 2), UPEC76 (C; lane 1), UPEC76Iha-CFT073 (B, 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.

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). 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)

<table>
<thead>
<tr>
<th>Parent Site cultured</th>
<th>Total No.</th>
<th>Parent &gt; Δiha-CFT073 mutant</th>
<th>Δiha-CFT073 (parent)</th>
<th>p&lt;sup&gt;+&lt;/sup&gt;</th>
<th>No. (%)&lt;sup&gt;−&lt;/sup&gt; with:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Parent only</td>
<td>Δiha-CFT073 mutant only</td>
<td>p&lt;sup&gt;+&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>CFT073&lt;sup&gt;−&lt;/sup&gt;R</td>
<td>Urine</td>
<td>19</td>
<td>12 (63)</td>
<td>2 (11)</td>
<td>&lt;0.02</td>
</tr>
<tr>
<td></td>
<td>Bladder</td>
<td>19</td>
<td>16 (84)</td>
<td>3 (16)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td></td>
<td>Right kidney</td>
<td>19</td>
<td>11 (58)</td>
<td>1 (5)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td></td>
<td>Left kidney</td>
<td>19</td>
<td>11 (58)</td>
<td>1 (5)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>UPEC76</td>
<td>Urine</td>
<td>24&lt;sup&gt;c&lt;/sup&gt;</td>
<td>17 (71)</td>
<td>5 (21)</td>
<td>0.017</td>
</tr>
<tr>
<td></td>
<td>Bladder</td>
<td>30</td>
<td>18 (60)</td>
<td>11 (37)</td>
<td>0.03</td>
</tr>
<tr>
<td></td>
<td>Right kidney</td>
<td>30</td>
<td>19 (63)</td>
<td>7 (23)</td>
<td>0.03</td>
</tr>
<tr>
<td></td>
<td>Left kidney</td>
<td>30</td>
<td>16 (53)</td>
<td>8 (27)</td>
<td>0.03</td>
</tr>
</tbody>
</table>

<sup>a</sup> 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.

<sup>b</sup> Percentages do not sum to 100% because some cultures yielded no growth or growth of both competing strains.

<sup>c</sup> Urine was unavailable for six mice from the UPEC76 experiments.

<sup>d</sup> 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.
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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