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Representational Difference Analysis between Afa/Dr Diffusely Adhering *Escherichia coli* and Nonpathogenic *E. coli* K-12

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Diffusely adhering *Escherichia coli* strains harboring Afa/Dr adhesins (Afa/Dr DAEC) have been associated with diarrhea and urinary tract infections (UTIs). The present work is the first extensive molecular study of a Afa/Dr DAEC strain using the representational difference analysis technique. We have searched for DNA sequences present in strain C1845, recovered from a diarrheagenic child, but absent from a nonpathogenic K-12 strain. Strain C1845 harbors part of a pathogenicity island (PAI_{CFT073}) and several iron transport systems found in other *E. coli* pathovars. We did not find genes encoding factors known to subvert host cell proteins, such as type III secretion system or effector proteins. Several C1845-specific sequences are homologous to putative virulence genes or show no homology with known sequences, and we have analyzed their distribution among Afa/Dr and non-Afa/Dr clinical isolates and among strains from the *E. coli* Reference Collection. Three C1845-specific sequences (MO30, S109, and S111) have a high prevalence (77 to 80%) among Afa/Dr strains and a low prevalence (12 to 23%) among non-Afa/Dr strains. In addition, our results indicate that strain IH11128, an Afa/Dr DAEC strain recovered from a patient with a UTI, is genetically closely related to strain C1845.

Diffusely adhering *Escherichia coli* (DAEC) strains, which are characterized by their diffuse adherence pattern on cultured epithelial HeLa cells (56), have been recognized as the sixth class of diarrheagenic *E. coli* and appear as a heterogeneous group (12, 41). A subclass of DAEC strains harbors adhesins of the Afa/Dr family (including Afa-I, Afa-III, Dr, Dr-II, and F1845), which recognize the decay-accelerating factor (DAF, or CD55) as a common receptor (43). Afa/Dr DAEC strains are identified in epidemiological studies by hybridization to a specific probe, *daaC*, which is common to operons encoding Afa/Dr adhesins (5). In addition, a PCR assay to detect Afa/Dr strains has been described recently (35). Afa/Dr DAEC strains have been associated with diarrhea in children (20, 22, 24, 28). However, some studies have reported that Afa/Dr DAEC strains are found equally in children with and without diarrhea (1, 17). Discrepancies between epidemiological studies could be explained in part by age-dependent susceptibility (37). Unlike other diarrheagenic pathovars of *E. coli*, Afa/Dr DAEC strains are also important in urinary tract infections (UTI) (43).

The alterations induced by wild-type Afa/Dr DAEC strains on polarized host epithelial cells have been studied extensively, but these strains remain poorly characterized at the molecular level. Infection of polarized cultured human intestinal cells by Afa/Dr DAEC strain C1845, isolated from a patient with diarrhea, or IH11128, recovered from a patient with UTI, is followed by elongation of brush border microvilli resulting from rearrangement of cytoskeleton proteins (4, 47), alteration of tight-junction-associated proteins (46), and impairment of several brush border-associated enzymatic activities (45, 47). The Afa/Dr adhesin operon also encodes invasins, AfaD, and DraD (18). Afa/Dr DAEC strains invade epithelial cells at a low rate by CD55- and CD66e-independent mechanisms through interaction with the $\alpha_5\beta_1$ integrin and a pathway involving caveolae and dynamic microtubules (23, 25). A recent study has reported that 50% of DAEC strains hybridize with an *irp2* probe, which is part of the yersiniabactin operon, encoding a siderophore-dependent iron transport system (12). In addition, two diverse pathogenicity islands (PAIs) have been described for some DAEC isolates. First, a few DAEC strains contain a homologue of the locus of enterocyte effacement (LEE) pathogenicity island and exhibit pathogenic properties characteristic of enteropathogenic *E. coli* (EPEC) strains (3). Second, it has recently been shown that the pyelonephritogenic Afa/Dr DAEC strain EC7372 harbors a PAI similar to the one described for the uropathogenic strain CFT073 (PAI_{CFT073}) (26), which encodes the classical UTI determinants hemolysin and P pili (27). Other Afa/Dr strains carrying the *hly* and *pap* operons and a marker from PAI_{CFT073} have been described recently (32).

To explore Afa/Dr DAEC at the molecular level, we have used a genomic approach, representational difference analysis (RDA) (38, 59), to analyze Afa/Dr strain C1845, recovered from a child with diarrhea (5). We have identified sequences present in strain C1845 but absent from a nonpathogenic *E.*

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coli K-12 strain. Our results indicate that *E. coli* C1845 harbors part of the PAI_{CFT073} and genes from several iron acquisition systems that are common to other enteric bacterial species. We also recovered C1845-specific sequences homologous to putative virulence genes or with no homology to known sequences, and we have analyzed their distribution among Afa/Dr and non-Afa/Dr clinical isolates and among strains from the *E. coli* Reference Collection (ECOR collection) (44). This analysis allowed us to identify sequences that have a high prevalence in Afa/Dr strains and a low prevalence in non-Afa/Dr strains.

MATERIALS AND METHODS

Bacterial strains. Strains used for subtractive hybridization were the DAEC strain C1845 (O75:NM), harboring the fimbrial adhesin F1845, isolated from a child with diarrhea (5), and the nonpathogenic laboratory *E. coli* K-12 strain MG1655, whose genome has been sequenced (6). The uropathogenic DAEC strain IH11128 (O75:H5:K), harboring the Dr adhesin, was isolated from a patient with pyelonephritis (60). EC7372, harboring the Dr-II adhesin, was isolated from a patient with pyelonephritis (50). Prototypes of the different classes of diarrheagenic *E. coli* are the EPEC strain 2348/69 (36), the enterohemorrhagic *E. coli* (EHEC) O157:H7 strain EDL931 (Pasteur Institute collection), the enterotoxigenic *E. coli* (ETEC) strain H10407 (15), and the enteroaggregative *E. coli* (EAEC) strain 042 (40).

The ECOR collection encompasses 72 strains that are representative of the range of genotypic variation in the *E. coli* species as a whole (44). We used 42 strains from the ECOR collection that are representative of the four main phylogenetic groups: 10 strains from group A, 8 strains from group B1, 15 strains from group B2, 7 strains from group D, and 2 strains that do not belong to any of the four groups. We have included the three strains (ECOR 64, ECOR 50, and ECOR 37) that hybridize with the *daaC* probe (29).

Afa/Dr DAEC clinical isolates were from children with diarrhea, asymptomatic children, or patients with pyelonephritis. Twenty *E. coli* strains that tested positive by colony DNA hybridization to the *daaC* probe were isolated in Brazil as described previously (55). Ten strains were recovered from infants with acute diarrhea, and 10 strains were recovered from a control group of asymptomatic children. Twenty-five strains that tested positive by the *afa* PCR assay (35) were recovered from children with diarrhea in New Caledonia (21). In addition, we have included as a control 20 strains isolated from children with diarrhea in the same study (21) that are negative by the *afa* PCR assay. Twelve strains testing positive by the *afa* PCR assay were from patients with pyelonephritis (2).

Chromosomal DNA extraction. Bacterial genomic DNA was extracted as described previously (49).

RDA. Clones of DNA fragments present in the genome of *E. coli* C1845 but absent from *E. coli* MG1655 were prepared as described previously (59). Chromosomal DNA from strain C1845 was digested with the restriction endonuclease *Msp*I, *Tsp*509I, or *Sau*3AI. Three subtractive libraries, resulting each time from two rounds of subtraction using first-round adapters (R) and second-round adapters (J) (59), were obtained. For the *Msp*I library, adapters were RMsp10 (5′-CGGTCGGTGA-3′), RMsp24 (5′-CAGCCACTCTCCGACCTCTCACGA-3′), JMsp10 (5′-CGGGTTCATG-3′), and JMsp24 (5′-ACCGACGTCGAC-3′).

Analysis of clones from subtractive libraries. DNA from the subtractive libraries was cloned into the *Cla*I (*Msp*I library) or *Eco*RI (*Tsp*509I library) site of the pBluescript vector (Stratagene) and then transformed into *E. coli* DH5α. Clones for the *Sau*3AI library were obtained by using the pUC18 *Sma*I/BAP vector of the SureClone ligation kit (Amersham Pharmacia Biotech, Orsay, France) and competent cells of the TOPO TA cloning kit (Invitrogen, Carlsbad, Calif.). Inserts were amplified by PCRs performed on transformant colonies by using primers P1 (5′-CCCCTCGAGGTCGACGGTAT-3′) and P2 (5′-CCGCT CTAGAACTAGTGGAT-3′) for the *Msp*I and *Tsp*509I libraries and primers UP (5′-GTAAAACGACGGCCAGT-3′) and RP (5′-CAGGAAACAGCTATGAC-3′) for the *Sau*3a library.

(i) DNA sequencing. PCR fragments were sequenced by using the PRISM Ready Reaction Big Dye Terminator kit and an automated ABI PRISM 377 XL DNA sequencer (both from Perkin-Elmer Applied Biosystems, Courtaboeuf, France) according to the manufacturer's instructions. Sequences were analyzed by using the BLASTN and BLASTX computer programs at the National Center for Biotechnology Information (Bethesda, Md.).

(ii) DNA hybridization techniques. To check for specificity, the amplified difference product from the second subtraction round of each bank was labeled by random-primed incorporation of [α-^{32}P]dCTP and used as a probe against *Dra*I- and *Eco*RV-digested DNA from C1845 and MG1655 in Southern blot experiments.

To study the distribution of C1845-specific sequences, purified PCR fragments were labeled by using the ECL direct nucleic acid labeling and detection system (Amersham Pharmacia Biotech) according to the manufacturer's protocol. Colony blotting was performed by using Hybond-N+ membranes (Amersham Pharmacia Biotech) and the ECL detection system according to the manufacturer's protocol. For Southern hybridization, *Bam*HI-digested chromosomal DNA was applied to an agarose gel and transferred by capillarity onto Hybond-N+ membranes as described elsewhere (54). Hybridizations were performed at 42°C with the ECL kit. Detection by chemiluminescence was performed and revealed by use of X-Omat film (Kodak).

PCR. Colony PCR was carried out by using PCR Beads Ready To Go (Amersham Pharmacia Biotech) according to the manufacturer's protocol and the Gene Amp PCR system 2400 (Perkin-Elmer Applied Biosystems). Oligonucleotides used in PCR experiments are described in Table 1. PCR was performed as follows: after an initial denaturation (5 min at 94°C), samples were subjected to 30 cycles of amplification, each of which consisted of 30 s at 94°C, 30 s at 55°C, and 1 min at 72°C. The annealing temperature was lowered to 46°C to investigate the presence of C1845-specific sequences among Afa/Dr clinical isolates. PCR fragments were purified from agarose gels by using the Qiaex II gel extraction kit (Qiagen, Courtaboeuf, France).

Statistical analysis. Statistical significance between groups was tested by the χ^2 test.

Nucleotide sequence accession numbers. The sequences of the subtracted DNA fragments have been assigned GenBank accession numbers AZ935556 to AZ935604.

RESULTS

Production of libraries of DNA fragments of Afa/Dr DAEC strain C1845 not found in the genome of *E. coli* K-12. By using RDA, we subtracted the genome of the nonpathogenic *E. coli* K-12 strain MG1655, which has recently been sequenced (6), from that of the pathogenic strain C1845, harboring the F1845 adhesin. Three libraries were produced by using the *Msp*I, *Sau*3AI, and *Tsp*509I restriction enzymes. To confirm that the sequences amplified from the second round of subtraction were C1845 specific, the difference product of each bank was labeled and used as a probe against *Dra*I- and *Eco*RV-digested DNAs from C1845 and the K-12 strain. Strong reactivity was observed with the pathogenic strain C1845, whereas no signal was detected for the K-12 strain (data not shown). Accordingly, the background of nonspecific sequences was very low, since only 5.5% of the sequences recovered were found in the K-12 genome.

Altogether, 172 C1845-specific clones were isolated and sequenced. Of these, 118 clones were unique. Ninety percent of the clones showed significant homology to known sequences, among which, for example, the clone of the F1845 adhesin operon (clone T006) confirms the validity of the RDA method. About 45% of the C1845-specific sequences recovered from RDA had homology to various plasmids (F, R100, R64, pColIb-P9, pO157, and pKYM), including sequences involved in plasmid transfer (*tra*), plasmid replication, plasmid transposases, insertion sequences, and noncoding plasmid sequences (data not shown). In addition, 8% of the C1845-specific sequences exhibited homology to prophage sequences, including P2 sequences and sequences homologous to O157:H7 EDL933 prophages (data not shown). Plasmid and phage sequences might be associated with virulence sequences, but it is unlikely that these sequences themselves play a role in virulence. Table 2 summarizes the clones that showed significant homology with published sequences other than plasmid and phage sequences. Sequences homologous to PAIs or iron ac-

TABLE 1. Oligonucleotides used in this study for PCR experiments

Name	Sequence	Length of PCR fragment (bp)
L8-F	CAT CAT CCG CTC CAT GC	
L6-F	TTC ACG AAG TAA CGC CAG	
R15-F	GAT TGC TGG GAA GGC TGG	
papF-F	TCG TTG CTT CTG ACA TCG G	470
papF-R	AAT CAT GCT CAT ACT GGC C	
shuA-F	CCG ATC TGC TGC GTC ATG	460
shuA-R	ATG GAC TCG TCA TTC GGC	
daaC-F	AAG GGG GTG GAC CTG AC	2,380
daaC-R	AGA CGG TAA TCC GCA TG	
M008-F	GGT TAT CTT CGG TAT C	250
M008-R	AGT TAG TGG ACT ACA GC	
M014-F	GAT CTA TGG CAA GAC GAG	240
M014-R	TCG ACA CAA TTC ACA GAC	
M020-F	CTT GTC ATC GGT ACC GAG	172
M020-R	CCA CAC TAT GCA GCC AGC	
M030-F	GAT CAG CGC CAC AAT TAC	120
M030-R	CAG TGG AGT CTG CTG CTC	
S014-F	AAG GAG TCA TGG CTG CTG	150
S014-R	TGC TGT AAC TGG TCG CGG	
S044-F	TCT GAC TAC TGC GAC AGG	220
S044-R	TCT GAG TAG CCG CAG TCG	
S070-F	TAT CAC GAA TCA GCT CAC	130
S070-R	GAG CTA TGT GAG CAA TAC	
S081-F	CTG TCT GAG ACT GTA GCG	120
S081-R	AGC CAT AAA GAG CTT GGC	
S094-F	ACC TTC GGG CAG GTT TTC	190
S094-R	ACC TGT TGT TTA ACG ACC	
S109-F	CAC AAC CTG TAG CTG CTG	65
S109-R	GTG CCG GAA CGT CAC TTC	
S111-F	AAT CCT TGA ATA TTG CTG	220
S111-R	AGC AGA CGA CAA GTT ATG	
S164-F	TCA TTA TTC GTG ACA GGC	60
S164-R	GAC GCG GGT AAT TTA TCC	
S177-F	TTG GTA TCT GCA TCG CCG	220
S177-R	CTT GAA GAT GAA ATT ACC	
S184-F	ACG CCG TAT TAT GTG CAG	133
S184-R	AAT AAC TGA GTG TCG ACC	
S199-F	ATC CTG CCG ACA ACG GTC	150
S199-R	CAG GGT TCT GAG TTC ATG	
T007-F	TGC ACC AGA ATA CAC GTC	192
T007-R	AGC TTC ATG TAG TGA GCG	
T018-F	CGC GTA GCG ACC AGT AGC	110
T018-R	CAA TAA TGG TGA AGG	
T011-F	CTG TGC GGC GCA GCG ATC	150
T011-R	TGG CCG GTA GGA TGA ATG	
T024-F	TGA TTC GGA TTG TGA TG	170
T024-R	ATC ACC TGC CGC TGA C	
T027-F	ATG GGC TCA TCT TCA ACG	140
T027-R	GCG AGA GCT ATG GCT TGG	
T033-F	ACG GAT AGG ACT GAT CAG	217
T033-R	GCG CTA ATG GAT CAG ATG	
T034-F	GTA TCA CAT ATC CTG TTG	260
T034-R	ATT CGT CAC TGA GCG CTG	

quisition systems are described in more detail below. A few sequences showed homology to putative virulence genes encoding a putative toxin (M008) or putative proteins involved in fimbrial assembly (S164 and S184). Finally, less than 10% of the C1845-specific clones had no homology with published sequences (Table 2). However, some sequences (S064, S094, S109, T007, and T027) were homologous to sequences from the genome projects of *E. coli* RS218 and the pyelonephritogenic *E. coli* strain CFT073 (available at www.genome.wisc.edu).

***E. coli* C1845 harbors part of PAI_{CFT073}.** A uropathogenic Afa/Dr DAEC strain, EC7372, which harbors a PAI similar to the one described for the pyelonephritogenic strain CFT073 (PAI_{CFT073}), has been described recently (26). PAI_{CFT073} encodes hemolysin (*hly*), P pili (*pap*), and several genes of unknown function (27). One gene, R4, is identical to *iha*, encoding a novel adhesin identified in *E. coli* O157:H7 (58). RDA revealed that *E. coli* C1845 also harbors several PAI_{CFT073} genes, since sequences homologous to the L6 (ModD), R3, R4, R6, and R12 open reading frames (ORFs) were recovered (Table 2; Fig. 1). We investigated the extent of similarity between C1845 sequences and PAI_{CFT073} by performing PCR with several pairs of primers covering different areas of PAI_{CFT073} (Fig. 1). Using primers specific for the left and right junctions of PAI_{CFT073} (34), primers specific for L6 and L8 (Table 1), and primers specific for R9 and R15 (26), we amplified fragments of the expected sizes from strain C1845 (Fig. 1). However, strain C1845 does not contain the entire PAI_{CFT073}, since we failed to amplify DNA fragments by using oligonucleotides complementary to the *hlyA*, *hlyD*, *hp1-hp4*, *papG*, and *papF* sequences (26, 31) (Fig. 1). We have confirmed by Southern blot experiments that C1845 DNA does not hybridize with *hlyA* or *papF* probes derived from EC7372 DNA (Fig. 2); on the other hand, a hybridization signal was detected with a *papA* probe (8) (Fig. 2). The PapA subunit is polymorphic (11 variants), and we have shown that a fragment of the expected size can be amplified from strain C1845 by using primers specific for the F10 *papA* allele (33), indicating the presence of a remnant of the *pap* operon. In addition, a fragment of the expected size can be amplified from strain C1845 by using primers specific for the F10 *papA* allele and R15 (Table 1). Taken together, these results indicate that *E. coli* C1845 harbors part of PAI_{CFT073}, including the R4 gene, but lacks most of the central region encoding the *hly* and *pap* operons.

On the other hand, none of the C1845-specific sequences identified by RDA matched with sequences from the LEE island, which includes a type III secretion system, intimin (Eae), and its receptor (Tir). In addition, genomic DNA from strain C1845 showed no hybridization in a Southern experiment with two different *eae* probes (7, 52) derived from EPEC DNA (Fig. 2) and showed no DNA amplification in a PCR experiment with degenerated oligonucleotides designed for type III secretion system detection (16). These results indicate that *E. coli* C1845 does not harbor the LEE island and suggest that C1845 does not encode a type III secretion system.

***E. coli* C1845 encodes several iron acquisition systems.** Pathogenic bacteria have adapted to the host iron-limiting environment by developing a variety of iron assimilation systems. A recent study has reported that strain C1845 harbors *irp2*, which is part of the yersiniabactin operon, encoding a siderophore-dependent iron transport system (12). Three DNA sequences recovered from RDA, S057, T011, and T035, exhibited significant homology to other iron acquisition systems found in pathogenic enteric bacteria (Table 2). Clone S057 carries the sequence of the *iucB* gene, which is part of the aerobactin operon. The siderophore aerobactin is produced by a variety of enteric bacteria, including *Shigella* spp. and some *E. coli* strains (13). Clone T011 is homologous to the *shuU* sequence. The *shu* operon, which allows the use of hemin as a

TABLE 2. Summary of BLAST search of *E. coli* C1845-specific clones

Clone[a]	Length (bp)	Sequence homology[b]	Probability	Accession no.
M003	308	*r12* (N), PAI of *E. coli* CFT073	$5e^{-14}$	AF081285
M008	314	Putative macrophage toxin (P), *E. coli* O157:H7	$7e^{-49}$	AAG54519
M014	320	D-Fructokinase (P), *E. coli* O157:H7	$6e^{-47}$	AAG57487
M020	323	2-Deoxy-D-gluconate 3-dehydrogenase (P), *E. coli* O157:H7	$6e^{-08}$	BAB37122
M022	281	Malonyl coenzyme A-acyl carrier protein transacylase (P), *Salmonella enterica* serovar Typhimurium	$2e^{-08}$	O85140
M028	398	ORF78 (P), putative transposase, EPEC	$2e^{-67}$	NP_053140
M029	310	ORF41 (P), putative transposase, EPEC	$8e^{-57}$	NP_053103
M030	292	ORF37 (P), hypothetical protein, 102-kb region of *Yersinia pestis*	$7e^{-28}$	CAA21360
S013	234	Transposon Tn*4311* (N), *E. coli*	$2e^{-71}$	M22041
S010	165	*r6* (N), PAI of *E. coli* CFT073	$4e^{-47}$	AF081285
S014	217	Putative protease (P), *E. coli* O157:H7	$1e^{-26}$	AAG54523
S044	140	*fliC* gene for flagellin (N), *E. coli* U4-41	$1e^{-52}$	AB028473
S057	175	*iucB* (N), aerobactin, *Shigella flexneri*	$3e^{-91}$	AF141323
S058	371	Noncoding downstream *pssA* (N), *E. coli* 413189-1	$2e^{-92}$	Y13614
S064	136	None		
S070	269	None		
S071	186	*r3* and *malX* (N), PAI of *E. coli* CFT073	$2e^{-51}$	AF081286
S076	286	Tn*3* transposase (P), *E. coli*	$1e^{-34}$	P03008
S077	184	Probable transposase (P), *Y. pestis*	$9e^{-23}$	T14971
S080	264	Tn*3* transposase (P), *E. coli*	$1e^{-12}$	P03008
S081	235	None		
S083	225	None		
S094	198	Z0251 (P), hypothetical protein, *E. coli* O157:H7	$6e^{-14}$	AAG54520
S097	232	None		
S099	294	Tn*3* transposase (P), *E. coli*	$1e^{-41}$	P03008
S109	85	None		
S111	248	Clone SauE4.C10 (N), *E. coli* K1 C5	e^{-130}	AF222134
S137	199	*r3* (N), PAI of *E. coli* CFT073	$1e^{-90}$	AF081286
S141	286	Tn*3* transposase (P), *E. coli*	$2e^{-40}$	P03008
S164	73	Putative fimbrial chaperone protein (P), *E. coli* O157:H7	$2e^{-28}$	AE005354
S165	283	IS*200* transposase (P), serovar Typhimurium	$3e^{-44}$	Q57334
S166	219	None		
S170	161	IS*100* transposase (P), *Y. pestis*	$5e^{-20}$	T17450
S177	239	None		
S184	201	TsaC (P), serovar Typhimurium	$1e^{-6}$	BAA82271
S199	214	None		
T002	242	ORF33 (P), hypothetical protein, 102-kb region of *Y. pestis*	$3e^{-29}$	CAA21356
T006	259	Upstream *daaE* (N), F1845 adhesin operon, *E. coli* C1845	e^{-139}	M27725
T007	201	None		
T011	210	ShuU (P), heme utilization, *Shigella dysenteriae*	$6e^{-32}$	AAC27812
T018	208	Sucrose hydrolase, invertase (P), *Erwinia amylovora*	$9e^{-17}$	CAC14601
T024	262	Z0259 (P), hypothetical protein, *E. coli* O157:H7	$4e^{-22}$	AAG54528
T027	206	None		
T028	441	Clone TspE4.A11 (N), *E. coli* K1 C5	e^{-151}	AF222183
T033	235	Clone SauE4.E6 (N), *E. coli* K1 C5	$3e^{-98}$	AF222140
T034	495	R4 (P), exogenous ferric siderophore receptor, PAI of *E. coli* CFT073; Iha (P), adhesin, *E. coli* O157:H7	$5e^{-34}$	AAC61730 AAF36432
T035	252	Putative molybdenum transport protein (P), *E. coli* O157:H7	$2e^{-41}$	AAG56050
		ModD (L6) (P), molybdenum transport protein, PAI of *E. coli* CFT073	$1e^{-24}$	AAC61710
T037	256	Syringomycin synthetase (P), *Pseudomonas syringae*	$1e^{-10}$	T14593
T039	269	Hypothetical protein PhuW (P), *Pseudomonas aeruginosa*	$1e^{-13}$	AAC13284

[a] Clones are designated by the initial letter of the restriction enzyme used (M, S, or T) and an alphanumeric designation.
[b] Only homologies with at least a probability of e^{-5} were retained. Homologies to plasmids ($n = 58$) and phages ($n = 11$) are not shown. N, similarity at the nucleotide level; P, similarity at the protein level.

carbon source, has been identified in *Shigella* spp. and is found in pathogenic *E. coli* strains (*chu* locus) (39, 63). We have shown by PCR using specific primers that another gene of the *shu* operon, *shuA* (Table 1), is also present in strain C1845. Clone T035 is homologous to a putative molybdenum transport protein encoded by PAI_{CFT073}. In addition, we have shown by using specific oligonucleotides (30) the presence in *E. coli* C1845 of a putative virulence gene, *iroN*, encoding a siderophore catechole receptor that is prevalent among *E. coli* isolates from patients with UTI or bacteremia (30, 53). Hence, strain C1845 contains multiple genes involved in iron acquisition systems which probably play roles during host infection.

Distribution of C1845-specific sequences among Afa/Dr and non-Afa/Dr clinical strains and strains from the ECOR collection. We have investigated the distribution of several C1845-specific sequences, including putative virulence genes and sequences with no homology to sequences in the databases, among *E. coli* Afa/Dr and non-Afa/Dr isolates and strains from

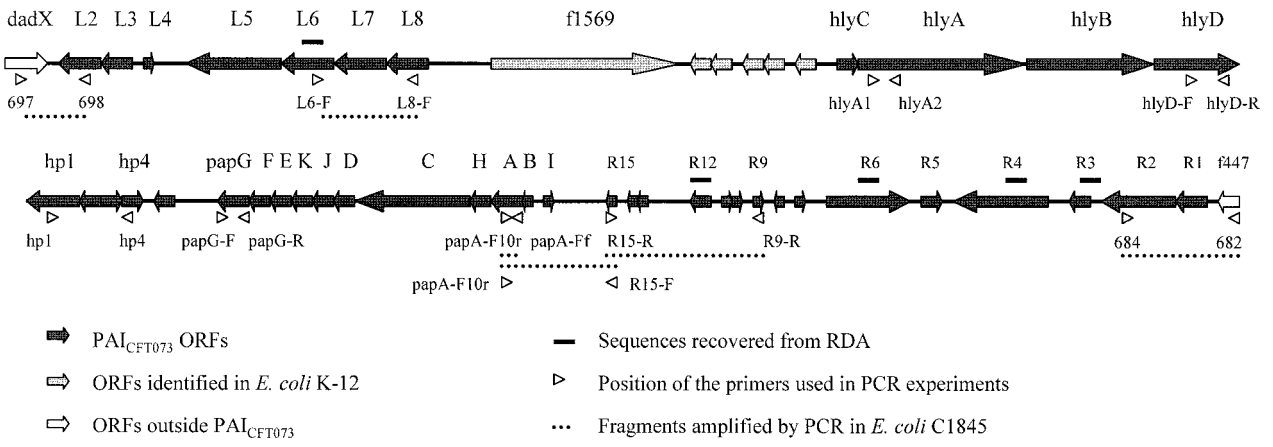

FIG. 1. *E. coli* C1845 harbors sequences from the left and right regions of PAI$_{CFT073}$. The schematic representation of PAI$_{CFT073}$ is derived from the work of Guyer et al. (reprinted from reference 27 with permission). Sequences homologous to the L6, R12, R6, R4, and R3 ORFs were recovered by RDA (Table 2). Positions of primers complementary to PAI$_{CFT073}$ sequences used in PCR experiments are indicated. Dotted lines, strain C1845 fragments amplified by PCR.

the ECOR reference collection. We have used 20 Afa/Dr *E. coli* isolates recovered from children with or without diarrhea in Brazil, 25 Afa/Dr *E. coli* isolates recovered from children with diarrhea in New Caledonia, and 14 Afa/Dr *E. coli* isolates recovered from patients with pyelonephritis (see Materials and Methods). As a control, we have used strains from the ECOR collection and non-Afa/Dr clinical isolates recovered from children with diarrhea in New Caledonia. We have chosen 42 strains from the ECOR collection, including the 3 strains which hybridize with the *daaC* probe (29). ECOR strains belong to four main phylogenetic groups (A, B1, B2, and D), and most of the pathogenic *E. coli* isolates are concentrated in groups B2 and D (29). Using specific oligonucleotides (11), we have shown by PCR that *E. coli* C1845 most likely belongs to the B2 group.

In a preliminary experiment, we investigated by PCR the presence of 22 C1845-specific sequences in the 20 Afa/Dr isolates from Brazil (data not shown). These sequences (oligonucleotides are described in Table 1) include putative virulence genes (M008, S164, S184, T011 [*shuU*], and T034 [*iha*]), putative metabolic genes (M014 and T018), and sequences with no homology to known proteins. Only four sequences had a high prevalence (>70%) among the Afa/Dr isolates (M030, S109, S111, and S164). The frequencies of the putative virulence sequences M008, S184, T011 (*shuU*), and T034 (*iha*) were 30, 25, 55, and 45%, respectively. Interestingly, some sequences (M008, S014, S094, and T024; M030 and S111; S081 and S184) had the same distribution, suggesting a genetic linkage. We then analyzed by colony hybridization on Afa/Dr and non-Afa/Dr strains the distribution of several sequences including M030, S109, S111, S164, and C1845-specific sequences with low or no homology with the published sequences that are not found in the databases of the *E. coli* RS218 and CFT073 genome projects (M020, S070, S081, S177, S184, S199, and T018). The results shown in Table 3 indicate that three clones (M030, S109, and S111) have a high prevalence among Afa/Dr strains and a low prevalence among non-Afa/Dr strains. These three clones did not hybridize with EPEC, EHEC, ETEC, or EAEC prototype strains (data not shown). In addition, these results confirmed that M030 and S111 have an identical distribution among *E. coli* strains.

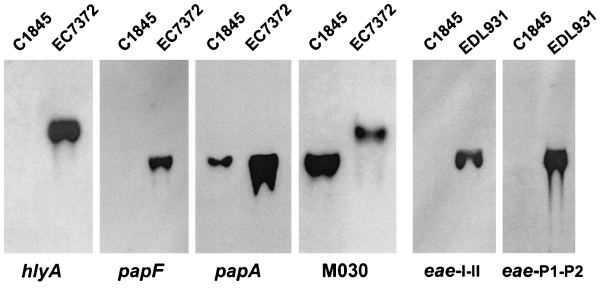

FIG. 2. Southern hybridization of *Bam*HI-digested DNA from *E. coli* C1845, EC7372, or EDL931 with *hly*, *pap*, or *eae* probes. Gene-specific probes were generated by PCR amplification on chromosomal DNA. *hlyA*, *papA*, and *papF* probes are derived from *E. coli* EC7372. Both *eae* probes are derived from *E. coli* 2348/69 (7, 52). Hybridization with a probe derived from *E. coli* C1845 (M030) is shown as a control.

Presence of C1845-specific sequences in the uropathogenic Afa/Dr DAEC strain IH11128. In in vitro experiments on cultured polarized epithelial cells, the structural and functional alterations induced by the pyelonephritogenic Afa/Dr DAEC strain IH11128 are similar to those induced by *E. coli* C1845 (45–47). It has been shown previously that strain IH11128, which harbors the Dr adhesin, does not carry classical uropathogenic *E. coli* virulence sequences such as *hly*, *cnf*, or *cdt* (26). To evaluate the similarity of strains C1845 and IH11128 at the molecular level, we investigated the presence of C1845-specific sequences recovered by RDA in strain IH11128 by PCR experiments or colony blotting (Table 3; also data not shown). *E. coli* IH11128 was found to harbor the diverse iron acquisition systems (*irp2*, *iucB*, *shuU-shuA*, and *iroN*) and the portion of the PAI$_{CFT073}$ that are found in strain C1845. In addition, like *E. coli* C1845, strain IH11128 harbors a remnant of the *pap* operon with the F10 *papA* allele. All 22 clones whose presence in Afa/Dr clinical isolates was investigated are

TABLE 3. Distribution of C1845-specific sequences among Afa/Dr and non-Afa/Dr strains

Strain origin[a]	No. of strains	No. (%) of isolates positive by colony hybridization[b] for:										
		M030	S109	S111	S164	M020	S070	S081	S177	S184	S199	T018
Afa/Dr												
ECOR	3	1 (33.5)	3 (100)	1 (33.5)	2 (66.5)	1 (33.5)	0 (0)	1 (33.5)	0 (0)	1 (33.5)	0 (0)	1 (33.5)
Diarrhea	35	30 (85.5)	28 (80)	30 (85.5)	24 (68.5)	10 (28.5)	0 (0)	12 (34.5)	0 (0)	12 (34.5)	0 (0)	5 (14.5)
Asymptomatic	10	8 (80)	6 (60)	8 (80)	5 (50)	2 (20)	0 (0)	1 (10)	0 (0)	1 (10)	1 (10)	2 (20)
Pyelonephritis	14	11 (78.5)	11 (78.5)	11 (78.5)	12 (85.5)	7 (50)	0 (0)	6 (43)	0 (0)	7 (50)	0 (0)	5 (35.5)
Total	62	50 (**80.5**[c])	48 (**77.5**[c])	50 (**80.5**[c])	43 (**69.5**)	20 (**32**[c])	0 (**0**)	20 (**32**)	0 (**0**)	21 (**34**)	1 (**1.5**)	13 (**21**)
Non-Afa/Dr												
ECOR	39	4 (10)	5 (13)	4 (10)	25 (64)	4 (10)	0 (0)	12 (31)	1 (2.5)	12 (31)	2 (5)	2 (5)
Diarrhea	20	3 (15)	9 (45)	3 (15)	8 (40)	1 (5)	0 (0)	4 (20)	0 (0)	4 (20)	1 (5)	1 (5)
Total	59	7 (**12**)	14 (**23.5**)	7 (**12**)	33 (**56**)	5 (**8.5**)	0 (**0**)	16 (**27**)	1 (**1.5**)	16 (**27**)	3 (**5**)	3 (**5**)

[a] The Afa/Dr ECOR strains are ECOR 64, ECOR 50, and ECOR 37. Afa/Dr clinical isolates from patients with diarrhea include 10 strains from Brazil and 25 strains from New Caledonia (35, 55). The 10 Afa/Dr strains from asymptomatic children were from Brazil (55). Afa/Dr clinical isolates from pyelonephritis patients include 12 strains positive by the *afa* PCR assay (35), IH11128, and EC7372. The non-Afa/Dr clinical isolates from children with diarrhea (New Caledonia) were negative by the *afa* PCR assay (35).

[b] PCR products amplified with specific oligonucleotide couples (Table 1) were used as probes. Numbers in bold show the percentages of positive isolates for all 62 Afa/Dr strains and all 59 non-Afa/Dr strains.

[c] The difference between the distribution of the sequence in Afa/Dr strains and that in non-Afa/Dr strains was significant ($P < 0.01$ by the χ^2 test).

present in strain IH11128, with the exception of three clones (S070, S177, and S199) that seem restricted to strain C1845, since they were absent or very rare among Afa/Dr clinical isolates (Table 3). Taken together, these results indicate that *E. coli* C1845 and IH11128 are closely related at the molecular level.

DISCUSSION

In the present study, we provide the first extensive molecular analysis of an Afa/Dr DAEC strain. Using the RDA approach, we have identified sequences from the genome of the Afa/Dr DAEC strain C1845, harboring the F1845 adhesin and recovered from a child with diarrhea, that are absent from the nonpathogenic *E. coli* K-12 strain MG1655. *E. coli* C1845 harbors several iron acquisition genes, genes from PAI_{CFT073}, and other putative virulence genes. However, our results indicate that C1845 does not harbor genes encoding factors known to subvert host cell proteins to induce brush border lesions, such as type III secretion system and effector proteins. An epidemiological approach allowed us to identify sequences that have a high prevalence in Afa/Dr strains and a low prevalence in non-Afa/Dr strains. Our results indicate that diarrheagenic *E. coli* C1845 is very close to Afa/Dr DAEC uropathogenic isolates.

Because iron is essential for bacterial growth and free iron is limiting within the host, bacterial pathogens have acquired diverse systems to acquire iron. In addition to the yersiniabactin siderophore (*irp2*), we have shown that *E. coli* C1845 harbors sequences encoding several iron transport systems found in other pathotypes of *E. coli*, including the aerobactin siderophore (*iuc*), a catechole siderophore receptor (*iroN)*, a heme transport system (*shu*), and a molybdenum transport system (*modD*). However, the reason for the redundancy of iron acquisition systems is unclear, and it has been postulated that the yersiniabactin system might have an alternative function, other than iron acquisition, in pathogenic *E. coli* strains (57). In addition, the diverse systems might play roles at different stages of the infection.

Several PAIs, which contribute to the rapid evolution of bacterial pathogens, have been described for pathogenic *E. coli* strains (14). We have shown that strains C1845 and IH11128 harbor the left and right ends of a PAI identified in a pyelonephritogenic *E. coli* strain, PAI_{CFT073} (27), but not the middle part of the island, encoding *hly* and *pap* operons. However, a remnant of the *pap* operon, carrying the F10 *papA* allele, was detected. Interestingly, the presence of a partial copy of the *pap* operon, with the F10 *papA* allele, has been found in several urosepsis strains belonging to the same serogroup as C1845 and IH11128, O75 (33), suggesting that this genetic organization is commonly found among O75 strains. One can hypothesize that the entire PAI_{CFT073} has been inserted into these strains and that a deletion has subsequently taken place. Whether the internal region of the PAI has been simply deleted or replaced by different genetic material remains unknown. Among the PAI_{CFT073} ORFs present in *E. coli* C1845 and IH11128, R4 is of particular interest because its translated sequence is identical to a novel adhesin of *E. coli* O157:H7, Iha, that mediates diffuse adherence to epithelial cells (58).

Besides iron acquisition systems and PAI_{CFT073} genes, we have identified a few C1845-specific sequences that show homology to putative virulence genes (M008, S164, and S184). Except for S164, these clones have only a narrow distribution among the Afa/Dr clinical isolates tested. Clone S164, encoding a putative fimbrial chaperone protein, is frequently found in Afa/Dr clinical isolates but is not specific for Afa/Dr strains. It is of interest that M008 is part of a group of four clones (M008, S014, S094, and T024) that are likely to be linked on the C1845 chromosome, because they exhibit the same distribution among the diverse *E. coli* strains and show homology to O157:H7 sequences that are part of an island not found in *E. coli* K-12 MG1655 (48). Sequences highly homologous (>98%)

to these clones are also found in the database of the RS218 genome project. Because this group of clones encodes a putative virulence factor, it may be part of a novel PAI that would be present in diverse *E. coli* pathovars. In addition, we have identified three sequences (M030, S109, and S111) that appear with a high frequency among Afa/Dr *E. coli* strains and a low frequency among non-Afa/Dr *E. coli* strains. These three Afa/Dr-specific sequences are found with similar frequencies in Afa/Dr isolates recovered from patients with diarrhea or pyelonephritis. Our results indicate that M030 and S111 are genetically linked, because they exhibit identical distribution among 121 *E. coli* strains. M030 exhibits low homology to an ORF of unknown function (ORF37) encoded by the 102-kb region of *Yersinia pestis* (9). Sequences highly homologous to M030, S109, and S111 are found in the database of the genome project for RS218, a K1 derivative responsible for meningitis, but these clones do not hybridize with EPEC, EHEC, ETEC, or EAEC prototype strains. Because of the high homology with RS218 sequences, these clones could encode factors that are important for both Afa/Dr and K1 pathogenic strains.

Afa/Dr DAEC strains have been associated with both diarrheagenic and uropathogenic infections. Recent data indicate that AfaE1, AfaE2, AfaE3, and F1845 adhesins are found in both diarrheagenic and uropathogenic human isolates (35). While strain C1845 was recovered from a child with diarrhea (5), our results indicate that this strain has several characteristics that have been associated with extraintestinal *E. coli* strains. These include the B2 phylogenetic group (51), the O75 serotype (42), the production of aerobactin (10), the presence of *iroN* (53), and the presence of sequences from PAI_{CFT073} (27). On the other hand, multilocus enzyme electrophoresis indicated that Afa/Dr DAEC strain C1845, as well as other DAEC strains, is phylogenetically close to EAEC strains (12); however, the phylogenetic relationship between DAEC strains isolated from patients with diarrhea and DAEC strains isolated from patients with UTIs has not been studied. To further investigate the relation of *E. coli* C1845 to uropathogenic isolates, we investigated the presence of C1845-specific sequences in the Afa/Dr strain IH11128, recovered from a patient with pyelonephritis. Despite their different origins, both strains appear very similar at the molecular level. The observation that *E. coli* C1845, isolated from a child with diarrhea, is very close to uropathogenic isolates is in agreement with the hypothesis developed by Germani et al. (19) that a single Afa/Dr strain can be responsible for diarrhea or UTI and/or with the hypothesis that isolation of Afa/Dr DAEC strains from patients with diarrhea may be related to the presence of uropathogenic *E. coli* in the colon without a causative link to the disease state (17).

It has recently been extensively documented that many enterovirulent bacteria subvert functional membrane-bound proteins as receptors to colonize epithelia and exploit the cell-signaling pathways to cross talk with the host cells and cause disease. The prototype of such subversive enterovirulent pathogens is EPEC, which colonizes the intestinal epithelium by translocating bacterial proteins through a type III secretion system to activate a signaling pathway within the underlying cell and cause the reorganization of the host actin cytoskeleton (61). Like EPEC, wild-type Afa/Dr DAEC strains promote dramatic lesions in the brush border of cultured human differentiated Caco-2 intestinal cells (4, 47). Some DAEC strains contain a homologue of the LEE PAI, which encodes a type III secretion system, and exhibit pathogenic properties characteristic of EPEC strains (3, 62). Our results indicate that strain C1845 does not encode the LEE PAI and lacks type III secretion system and effector proteins, implying that the brush border cytoskeleton is altered by a mechanism different from the one developed by EPEC strains. No known virulence factors involved in cytoskeleton injuries have been found in the RDA conducted in this study, suggesting that interaction between the Afa/Dr adhesins and membrane-bound receptors is the major mechanism by which Afa/Dr DAEC pathogens subvert host cell-signaling pathways to develop pathogenicity. This interpretation is consistent with previous results (47) and with recent data showing that interaction of Afa/Dr DAEC adhesins with CD55 induces the transepithelial migration of polymorphonuclear leukocytes in human intestinal T84 cell monolayers and a proinflammatory response. This phenomenon follows the adhesin-dependent tyrosine phosphorylation of several T84 proteins and activation of the mitogen-activated protein kinases (F. Bétis, P. Brest, V. Hofman, J. Guignot, M.-H. Bernet-Camard, B. Rossi, A. L. Servin, and P. Hofman, unpublished data).

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