# Characterization of the AfaD-like family of invasins encoded by pathogenic *Escherichia coli* associated with intestinal and extra-intestinal infections

Marie-Isabelle Garcia[a,1], Mabel Jouve[a,b], James P. Nataro[c], Pierre Gounon[b], Chantal Le Bouguénec[a,*]

[a] *Unité de Pathogénie Bactérienne des Muqueuses, Institut Pasteur, 28 rue du Docteur Roux, 75724 Paris Cedex 15, France*
[b] *Station Centrale de Microscopie Electronique, Institut Pasteur, 28 rue du Docteur Roux, 75724 Paris Cedex 15, France*
[c] *Center for Vaccine Development, University of Maryland School of Medicine, 10 S Pine Street, Baltimore, MD 21201, USA*

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**Abstract The afimbrial adhesive sheath, encoded by the *afa-3* gene cluster, is composed of two proteins with different roles in bacterium–HeLa cell interactions. AfaE is required for adhesion and AfaD for internalization. In this study, we found that the AfaD invasin was structurally and functionally conserved among human *afa*-expressing strains, independently of AfaE subtype and clinical origin of the *Escherichia coli* isolate. The AggB protein from enteroaggregative *E. coli* was also found to be an AfaD-related invasin. These data suggest that AfaD is the prototype of a family of invasins encoded by adhesion-associated operons in pathogenic *E. coli*.** 

*Key words:* AfaD; Invasin; Diffusely-adherent *Escherichia coli*; Enteroaggregative *Escherichia coli*; Urinary tract infection; Diarrhea

## 1. Introduction

The *afa* gene clusters encode an afimbrial adhesive sheath produced by *Escherichia coli* strains isolated from human patients with urinary tract infections or diarrhea [1–6] and from animals with extra-intestinal infections [7]. The adhesive sheath is composed of two proteins, AfaD and AfaE, which are independently exposed at the bacterial cell surface [8]. We have shown that bacterium–epithelial cell interactions involve a two-step mechanism in which AfaE is required for bacterial adhesion to HeLa cells and AfaD for the uptake of adherent bacteria into these cells [9]. Due to the heterogeneity of the *afaE* gene, various AfaE adhesins (or AfaE subtypes), differing in biochemical and antigenic properties, are produced by human clinical *E. coli* isolates [10]. Several operons of the *afa* family (including *afa-1*, *afa-2*, *afa-3*, and *afa-5*), encoding various AfaE proteins (AfaE-I, AfaE-II, AfaE-III, and AfaE-V, respectively) have been cloned from diarrhea-associated and uropathogenic *E. coli* strains ([4,5], Le Bouguénec unpublished data). The *afa* family of gene clusters also includes the *daa* and *dra* operons, which encode the fimbrial F1845 and Dr AfaE-related adhesins [11,12]. Although structurally different, these adhesins all mediate the diffuse adhesion of bacteria to epithelial cells and it has been shown that most (AfaE-I, AfaE-III, Dr, and F1845) recognize the same receptor, the decay-accelerating factor (DAF) [13]. The structure–function relationships of AfaE adhesins have been studied extensively, but very little is known about those of the AfaD invasin. We assessed the structural and functional conservation of the AfaD invasin by comparing the proteins produced by pathogenic *E. coli* strains associated with intestinal and extra-intestinal infections and expressing various *afaE* genes.

## 2. Materials and methods

### 2.1. Bacterial strains and plasmids

We studied 13 *E. coli* strains originating from human intestinal or urinary tract infections. *E. coli* A30, KS52, A22, 6290, AL643 and AL657 were from a collection of uropathogenic *E. coli* strains [10]. *E. coli* AL856, AL851, 3127N, 1444N and 2979N were from a collection of diarrhea-associated *E. coli* strains isolated in Noumea, New Caledonia [1]. Diffusely adherent *E. coli* C1845 was isolated from an infant with diarrhea [12]. The enteroaggregative *E. coli* (EAggEC) strain, 17-2, is a Chilean pediatric diarrheal isolate [14]. Plasmid pBJN406 was used as a source of *dra* sequences [11]. *E. coli* HB101 was used as a host for molecular cloning [15]. Recombinant *E. coli* M15 harboring the *lac*I-containing plasmid, pREP4, was used for the purification of $His_6$-tagged proteins (Qiaexpress kit, Qiagen). Vectors pILL570 [16], pACYC184 [17] and pQE30 (Qiaexpress kit, Qiagen) were used in cloning experiments. Recombinant plasmids are listed in Table 1. Culture conditions were as previously described [9].

### 2.2. DNA analysis and genetic techniques

To sequence *afaD* genes, the *afaD* region of the various strains was first amplified using 25 cycles of PCR (94°C for 1 min, 52°C for 1 min, and 72°C for 2 min in a Perkin-Elmer Cetus thermal cycler), with oligonucleotides 60 (5′-tgtacctgagcgggctg-3′) and 63 (5′-tcagttaatttatccgg-3′) derived from the published *afa-3* gene cluster sequence (accession number X76688, [18]) used as primers. The amplification products were sequenced with the Thermosequenase cycle sequencing kit (Amersham) and by Big Dye Terminator chemistry (Perkin-Elmer Applied Biosystems, Foster, USA). Sequences were screened for region of similarity using the GAP program of the GCG sequence analysis software package, version 7-UNIX. Multiple alignments were generated using the CLUSTAL W program at the National Center for Biotechnology Information.

### 2.3. Purification of $His_6$-tagged proteins and coating of carboxylated polystyrene beads with proteins

The $His_6$-AfaD-V, $His_6$-DaaD, and $His_6$-AggB proteins were puri-

*Corresponding author. Fax: (33)-1-40 61 36 40.
E-mail: clb@pasteur.fr

[1] Present address: Università di Roma 'La Sapienza', Dipartimento di biotecnologie cellulari ed ematologie, Sezione di genetica molecolare, Viale Regina Elena, 324, I-00161 Roma, Italia.

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Table 1
Recombinant plasmids used in this study

| Plasmid | Relevant characteristics | Source or reference |
|---|---|---|
| pILL1018 | 11.6-kb *Sau*3A fragment carrying the *afa-1* gene cluster from KS52 inserted into pILL570 | [18] |
| pILL1019 | 11.2-kb *Sau*3A fragment carrying the *afa-2* gene cluster from A22 inserted into pILL570 | this study |
| pILL1101 | 11.6-kb *Sau*3A fragment carrying the *afa-3* gene cluster from A30 inserted into pILL570 | [18] |
| pILL1128 | 8.5-kb *Bam*HI–*Hin*dIII fragment carrying the *daa* gene cluster from strain C1845 inserted into pBR322 | [8] |
| pILL1147 | 11.7-kb *Sau*3A fragment carrying the *afa-5* gene cluster from AL851 inserted into pILL570 | this study |
| pILL1168 | 10.5-kb *Bam*HI fragment carrying the *afa-3* gene cluster from A30 inserted into pBR322, into which a stop codon was introduced in the *afaD* gene | [8] |
| pILL1198 | *Bam*HI–*Hin*dIII PCR product from C1845 inserted into pQE-30 | this study |
| pILL1200 | *Bam*HI–*Hin*dIII PCR product from strain 17-2 inserted into pQE-30 | this study |
| pILL1202 | *Bam*HI–*Hin*dIII PCR product from pILL11147 inserted into pQE-30 | this study |
| pILL1218 | *Cla*I fragment (extracted from pJPN39Ω4 [27]) carrying the *agg* gene cluster from 17-2 in which the pilin-encoding gene *aggA* has been mutated by introduction of an Ω cassette inserted into pACYC184 | this study |
| pBJN406 | 12-kb *Hin*dIII fragment carrying the *dra* gene cluster from strain IH11128 inserted into pACYC184 | [11] |

fied with Ni-NTA resin under denaturing conditions, from induced cultures of M15(pREP4) containing the recombinant plasmids, pILL1202, pILL1198 and pILL1200, respectively, as described previously [8]. The purified proteins were dialyzed against distilled water. To maintain protein stability, the pH of the solutions was adjusted to 4 before storage at −20°C. Carboxylated microspheres 1 μm in diameter (Polysciences, Inc., Warrington, PA, USA) were coated with the various $His_6$-tagged proteins or bovine serum albumin (BSA) as previously described [9].

### 2.4. Adhesion and invasion assays

HeLa and CHO cells were maintained in culture as previously described [7,9]. For infection experiments, monolayers were seeded with $4\times10^5$ cells in 35-mm tissue culture dishes (Corning, Corning, NY, USA), incubated overnight, and washed with fresh medium. Each plate was then incubated with either $4\times10^7$ bacteria or 30 μl of a suspension of protein-coated polystyrene beads for 3 h at 37°C in 1 ml of tissue culture medium containing 1% D-mannose. The bacterial invasion of epithelial cells was assessed by determining resistance to gentamicin, a bacterial antibiotic, as previously described [9].

### 2.5. Microscopy

After the 3-h incubation, infected HeLa monolayers were washed with phosphate-buffered saline (pH 7.4), fixed and embedded in epoxy resin as previously described [9]. For light microscopy, semi-thin sections (2 μm) were examined following staining with 1% methylene blue and 1% Azur II in 1% borax. For transmission electron microscopy, ultrathin sections (80 nm) stained with uranyl acetate and lead citrate were examined with a JEOL 1010 transmission electron microscope operating at 80 kV.

For analysis of the entry of beads into CHO cells, fluorescent crimson carboxylated microspheres (Molecular Probes, catalogue no. F8816, Interchim, Montluçon, France) were used. Extracellular particles were labeled by incubation of the unpermeabilized fixed monolayers with rabbit anti-AfaD-III polyclonal antibodies [8] (for detection of AfaD-III, AfaD-V, and DaaD-coated beads) or rabbit anti-AggB polyclonal antibodies (kindly provided by Laure Plançon). The particles were washed three times and then incubated with FITC-labeled goat anti-rabbit IgG antibodies (Biosys, Compiègne, France). Samples were mounted and viewed with an epifluorescence microscope. Both intra- and extracellular beads were detected by the intrinsic red fluorescence of the beads whereas green fluorescence indicated extracellular beads only.

### 2.6. Nucleotide sequence accession numbers

The sequences of *daaD*, *afaD1*, *afaD2*, *afaD5*, and *draD* have been deposited in GenBank under the accession numbers AF233530–AF233533 and AF284829, respectively.

## 3. Results

### 3.1. The AfaD proteins are structurally conserved

The degree of conservation of *afaD* genes was first determined by sequencing PCR products from representative strains expressing the *afa-1*, *afa-2*, *afa-5*, *dra*, and *daa* gene clusters (strains KS52, A22, AL851, MC1061 (pJBN406), and C1845, respectively) and comparing the sequences obtained with that of the *afaD* gene from the *afa-3*-expressing strain, A30, which has been published [18]. A high degree of sequence similarity was found among these six sequenced *afaD* genes (identities of 93.02–100%), corresponding to amino acid (AA) sequence similarities of 91.89–100% for the deduced products (Fig. 1A). Minor point mutations were observed, principally within the first 21 N-terminal AA of the predicted mature products (Fig. 1A). To evaluate further the degree of conservation of the *afaD* gene, we first entirely sequenced the *afaD* gene, which encodes an AfaE-V adhesin, from strain 3127N, and then sequenced six additional PCR products in the region of variability (corresponding to AA 1–35 of the mature protein). These were obtained from diarrhea-associated or uropathogenic strains (AL856, 6290, 1444N, AL643, 2979N, and AL657), for which the *afaE* subtype had already been determined by PCR (*afaE* subtypes coding for the AfaE-III, AfaE-V, F1845, and AfaE-X adhesins)

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Fig. 1. Sequence alignment for the proteins of the AfaD family. Sequences of AfaD-like products from clinical isolates originating from patients with urinary tract infections (UTI) or diarrhea (D). The *afa*-related gene clusters carried by these strains encode various AfaE adhesin subtypes (I, II, III, Dr, V, X, F1845). AA sequences were deduced from the DNA sequences of the PCR products amplified from the *afaD* gene. Dots indicate AA identical to those of AfaD from A30. A: Alignment of the sequence of AfaD from the *afa-3*-expressing strain A30 with those of AfaD-like products from representative human *E. coli* strains expressing the *afa-1*, *afa-2*, *afa-5*, *dra* and *daa* operons. *The *draD* gene was sequenced using a product amplified by PCR from pBJN406. Numbering starts at the first AA of the AfaD peptide from A30. The predicted site of signal peptide cleavage for AfaD from A30 is indicated by a vertical arrow. B: Alignment of the 35 N-terminal residues of mature AfaD-like products (AA 27–51) from human diarrhea-associated and uropathogenic *E. coli* strains expressing various *afaE* subtypes with those of AfaD from strain A30. **Untypable *afaE* subtype. C: Multiple alignments of AfaD-III from A30, AfaD-VII and AfaD-VIII proteins encoded by the *afa-7* and *afa-8* operons from bovine *E. coli* isolates [7], and AggB and AafB encoded by the *agg* and *aaf* operons from enteroaggregative *E. coli* strains 17-2 and 042 [14,21]. Asterisks indicate identical residues.

[19,20]. The *afaD* sequence was highly conserved, regardless of AfaE subtype and clinical origin of the isolate (Fig. 1A,B). Recent protein sequence database searches have shown significant similarities between the AfaD-III produced by strain A30 and other proteins encoded by adhesion systems from pathogenic *E. coli*. Fig. 1C shows the similarity of these proteins to AfaD-III from A30 and to each other. The AfaD-VII and AfaD-VIII proteins produced by bovine clinical *E. coli* isolates, which have recently been demonstrated to mediate the uptake of these bacteria into HeLa cells, are 46.6 and 45.2% identical respectively to the representative AfaD invasin [7]. The AggB and AafB products, encoded by the *agg* and *aaf* adhesion operons from enteroaggregative *E. coli*, the function of which is unknown, are 50.7 and 58.3% identical to AfaD-

**A**

| Clinical isolate (adhesin) | Origin | 1 ↓ 50 |
|---|---|---|
| A30 (AfaE-III) | UTI | MNGSIRKMMRVTCGMLLMVMSGVSQAAELHLESRGGSGTQLRDGAKVATG |
| KS52 (AfaE-I) | UTI | .....N...L... |
| A22 (AfaE-II) | UTI | .....S...T...H.SR.G.....E.L... |
| AL851 (AfaE-V) | D | .....S...T...H.SR.G.....E.L... |
| 3127N (AfaE-V) | D | ..... |
| IH11128* (Dr) | UTI | ..... |
| C1845 (F1845) | D | .....N...L... |

| | | 51 100 |
|---|---|---|
| A30 (AfaE-III) | UTI | RIICREAHTGFHVWMNERQVDGRAERYVVQSKDGRHELRVRTGGDGWSPV |
| KS52 (AfaE-I) | UTI | ..... |
| A22 (AfaE-II) | UTI | ..... |
| AL851 (AfaE-V) | D | ..... |
| 3127N (AfaE-V) | D | ..... |
| IH11128* (Dr) | UTI | ..... |
| C1845 (F1845) | D | ..... |

| | | 101 147 |
|---|---|---|
| A30 (AfaE-III) | UTI | KGEGGKGVSRPGQEEQVFFDVMADGNQDIAPGEYRFSVGGACVVPQE- |
| KS52 (AfaE-I) | UTI | ..... |
| A22 (AfaE-II) | UTI | .....F.....V....N.....G... |
| AL851 (AfaE-V) | D | .....F.....G... |
| 3127N (AfaE-V) | D | ..... |
| IH11128* (Dr) | UTI | ..... |
| C1845 (F1845) | D | ..... |

**B**

| Clinical isolate (adhesin) | Origin | 27 51 |
|---|---|---|
| A30 (AfaE-III) | UTI | AELHLESRGGSGTQLRDGAKVATGRRIICREAHTGF |
| AL856 (AfaE-III) | D | ..... |
| 6290 (AfaE-V) | UTI | ..... |
| 1444N (F1845) | D | .....N...L..... |
| AL643 (F1845) | UTI | .....N...L..... |
| 2979N (AfaE-X**) | D | ..... |
| AL657 (AfaE-X**) | UTI | ..... |

**C**

| Clinical isolate | Invasin | |
|---|---|---|
| 17-2 | AggB | --MLKKS----ILPMSCGVLVMVMSGLLDAAEITLIS-HKTLGSQLRDGMKLATGRIACR |
| 042 | AafB | ---MKKG----MLSVSCGMLLMVISGLSQATEISLEGLHRNMGEQLFDGDILATGRIICR |
| A30 | AfaD-III | MNGSIRK----MMRVTCGMLLMVMSGVSQAAELHLES-RGGSGTQLRDGAKVATGRIICR |
| 239KH89 | AfaD-VIII | ---MKK-----IQIVCSGIVLVVISSLAQAVELSLNT-SDGRSGELKDGTKVATGRIICR |
| 262KH89 | AfaD-VII | ---MKKNGKTHVLHGCLAVVMVMMAGVSQAAELSLDV-RKAMGSELRDGERIATGRIICR |
| | | * * * * ** ***** ** |
| 17-2 | AggB | EPHDGFHIWINASQNG-KVGHYIVQNNRETKHELKVKIGGGGWSSSLIEGQRGVYRQGEE |
| 042 | AafB | ERHTGFHIQMNARQVEGRPGHYIVQGSKDTQSKLWVRLGREGWTSPTGGGQQGIVRSGQE |
| A30 | AfaD-III | EAHTGFHVWMNERQVDGRAERYVVQ-SKDGRHELRVRTGGDGWSPVKGEGGKGVSRPGQE |
| 239KH89 | AfaD-VIII | GTYTSFHIWMNSRQMGNIPGHYIILGRHDSHNEMRVRLDGAGWLPSV-SDGQGMVSTGIP |
| 262KH89 | AfaD-VII | EAHTGFHVWMNGREDEGRPGHLLIQGKHDSRNEIRARVEGEGWSAIT-EGEHRLVKAGEE |
| | | ** * ** * |
| 17-2 | AggB | KQAIFDIMSDGNQYSAPGEYIFSVSGECLISRG-- |
| 042 | AafB | EQVIFDVMADGNQWAKPGEYIFSVSGKCLTSWE-- |
| A30 | AfaD-III | EQVFFDVMADGNQDIAPGEYRFSVGGACVVPQE-- |
| 239KH89 | AfaD-VIII | EQHTFDVVIDGNQLLGPDEYILSVSGECS------ |
| 262KH89 | AfaD-VII | EQAVFDVVSDGRQRAGADEYVLSVSGSCVDSRSQE |
| | | * ** ** * ** * * * |

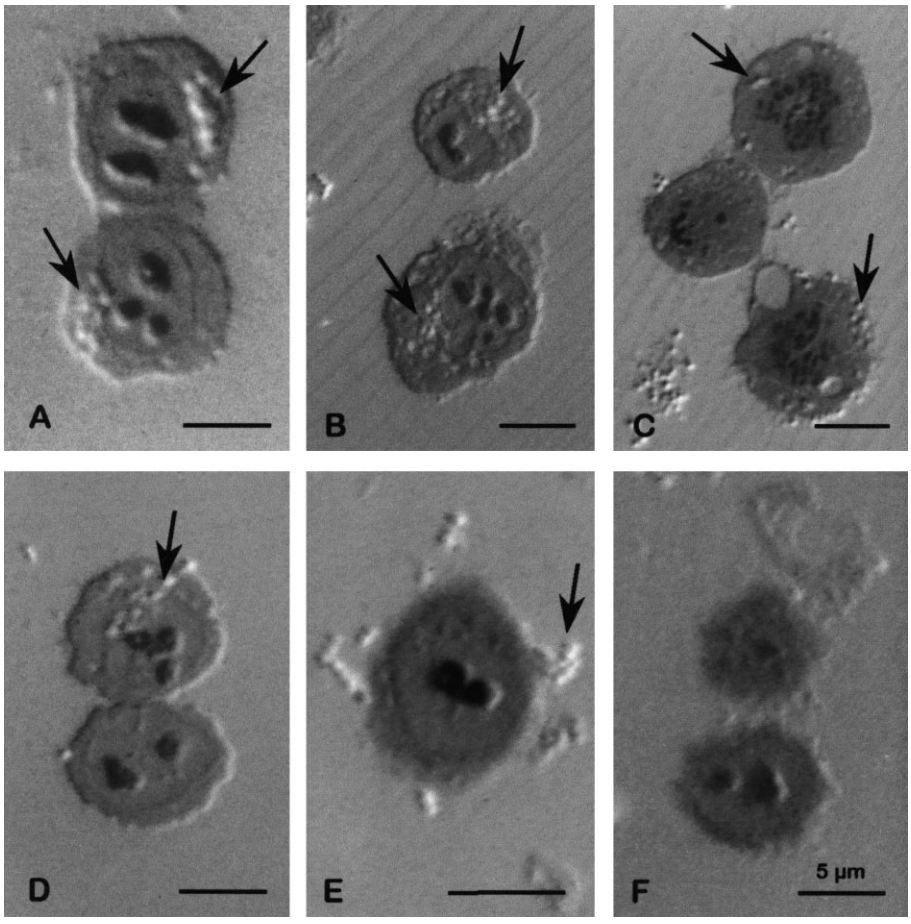


Fig. 2. Interaction of polystyrene beads coated with AfaD-like proteins with HeLa cells. Semi-thin sections (2 μm) of HeLa cells incubated for 3 h with polystyrene beads coupled to $His_6$-AfaD-like products purified from M15(pREP4) harboring the plasmid: (A) pILL1198, (B) pILL1202, (C) pILL1200. Beads coated with $His_6$-AfaD-III (D) were used as a positive control for internalization. Beads coated with $His_6$-AfaE-III (E) were used as a positive control for adhesion and as a negative control for internalization. Beads coated with BSA (F) were used as a negative control for both adhesion and internalization.

III, respectively [14,21]. The sequence similarities extend over the full lengths of these proteins with a highly conserved region of 8 AA (ATGRIXCR), near the N-terminus, containing a potential site for the phosphorylation by protein kinase C (ATGR).

### 3.2. *The AfaD-like products promote the uptake of carboxylated beads into HeLa and CHO cells*

We have previously demonstrated that the purified $His_6$-AfaD encoded by the *afa-3* gene cluster of A30 is sufficient to promote the entry of carboxylated polystyrene beads into HeLa cells [9]. We investigated whether the structural conservation of AfaD proteins was associated with their ability to promote invasion. AfaD-like products with AA sequences differing from that of the representative AfaD-III (DaaD from strain C1845 and AfaD-V from strain AL851) were tested for invasion. We also studied the function of the related AggB product. Sequences encoding the mature AfaD-like proteins of strains carrying the *daa*, *afa-5*, and *agg* operons were first amplified using the following primer pairs: oligonucleotides 69 and 70, 99 and 100, or 97 and 98 (Table 2). The PCR products were then inserted into pQE-30 as indicated in Table

Table 2
Oligonucleotides used for the purification of $His_6$-tagged proteins

| Oligonucleotide | | | Amplified sequences | | |
|---|---|---|---|---|---|
| Number | Strand | Sequence (5′→3′) | Strain used as template | Designation of the gene cluster | Characteristics of the restricted products |
| 69 | + | ttcgaaGGATCCgctgagctccacctggagagccgggg | C1845 | *daa* | mature DaaD coding region |
| 70 | − | ctacgAAGCTTgcgctttattcctgtggcaccacacag | | | |
| 99 | + | ttcgaaGGATCCgcgga(a,g)ctgacactggagagtcac | AL851[a] | *afa-5* | mature AfaD-V coding region |
| 100 | − | ctacgaAAGCTTttattcctgtggccccacacaggc | | | |
| 97 | + | ttcgaaGGATCCgcagaaattacgttgataagtcac | 17-2 | *agg* | mature AggB coding region |
| 98 | − | ctacgaAAGCTTtcatcctcttgatattagacattc | | | |

Restriction sites *Bam*HI (GGATCC) and *Hin*dIII (AAGCTT) used for the cloning are indicated in upper-case letters.
[a]Amplification was performed with the recombinant plasmid, pILL1147, as a template.

1. The resulting recombinant plasmids, pILL1198, pILL1202 and pILL1200, respectively, were introduced into the M15(pREP4) strain. Following induction with IPTG, the $His_6$-DaaD, $His_6$-AfaD-V and $His_6$-AggB fusion proteins were purified on Ni-NTA columns and covalently coupled to carboxylated microspheres 1 μm in diameter (see Section 2). Beads coated with $His_6$-DaaD, $His_6$-AfaD-V or $His_6$-AggB proteins were taken up by HeLa cells during a 3-h incubation, as were beads coupled to $His_6$-AfaD-III protein (Fig. 2). The internalization of beads was quantified by direct counting on semi-thin sections under a light microscope. The efficiency of entry was similar for all the AfaD-like-coated beads, with a mean of 3.4–5.2 intracellular beads per cell. In contrast, $His_6$-AfaE-III-coated beads associated with HeLa cells but did not enter them and beads coated with BSA did not interact with HeLa cells at all (Fig. 2).

Carboxylated polystyrene beads were also incubated for 3 h with CHO cells, which do not express DAF, the receptor of the AfaE adhesin. Intracellular and extracellular beads were identified by immunofluorescence (see Section 2). In contrast to $His_6$-AfaE-III-coated beads, which did not associate with CHO cells, beads coated with the four recombinant AfaD-like proteins were observed to interact with the cell surface and to be taken up into the CHO cells (data not shown), indicating that DAF is not a receptor for AfaD invasins.

### 3.3. *AfaD-producing strains are taken up by HeLa cells, independently of AfaE subtype*

After 3 h of infection, recombinant HB101(pILL1101), harboring a plasmid carrying the cloned *afa-3* gene cluster, has been demonstrated to adhere to and to invade HeLa cells (1.1% of total bacteria) [9]. We investigated whether adhesin subtype affected the adhesion and invasion properties of *afa*-expressing bacteria, by comparing the interaction of recombinant strains HB101(pILL1018), HB101(pILL1019), HB101(pILL1147) and HB101(pILL1128) (carrying the *afa-1*, *afa-2*, *afa-5* and *daa* gene clusters, respectively) with that of HB101(pILL1101). Transmission electron microscopy showed that all strains adhered similarly to HeLa cells. As shown in Fig. 3A,B for the *afa-5* and *daa*-expressing HB101 strains, adherent bacteria were surrounded by microvillar extensions of the cellular membrane. For all these recombinant strains, a low level of internalization into HeLa cells was observed, with a frequency similar to that of the previously studied HB101(pILL1101). Internalized bacteria remained within vacuoles, with no apparent multiplication during the 3 h of infection.

### 3.4. *AggB complements the invasion deficiency of an afa-3-expressing strain carrying a mutated afaD gene*

As the purified $His_6$-AggB protein directed the entry of beads into HeLa cells, we further investigated the role of

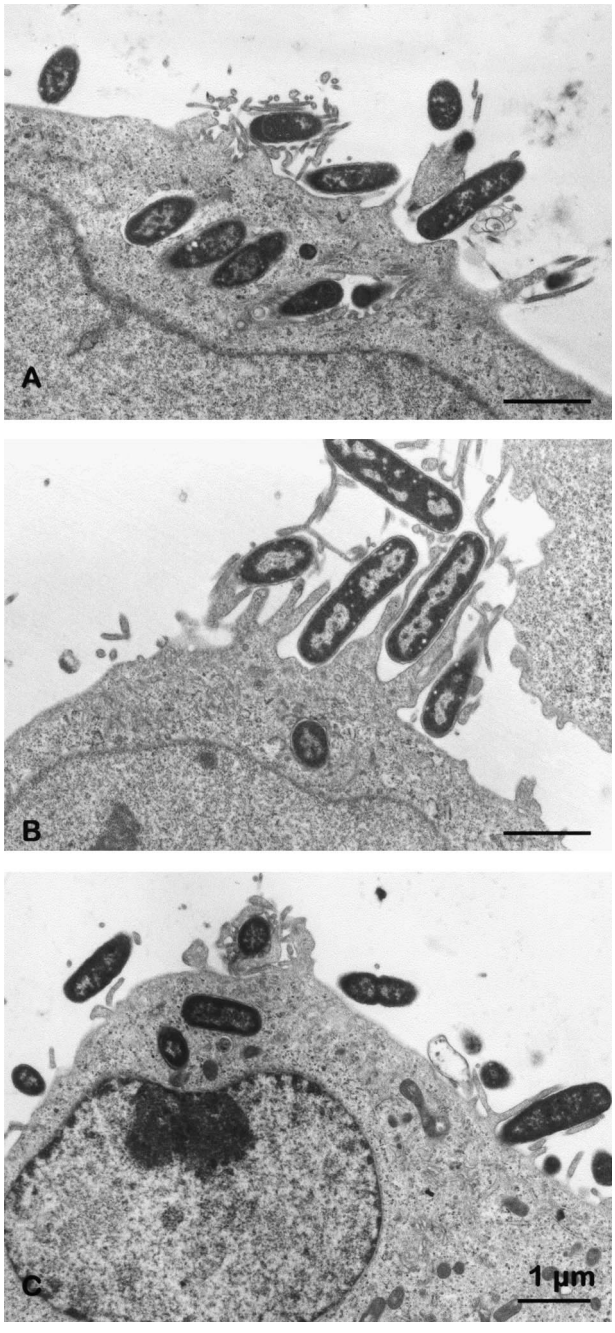


Fig. 3. Interaction of AfaD-like protein-producing strains with HeLa cells. Ultra-thin sections (80 nm) of HeLa cells incubated for 3 h with *E. coli*: (A) HB101(pILL1147) producing the AfaD-V and AfaE-V proteins, (B) HB101(pILL1128) producing the DaaD and DaaE proteins, (C) HB101(pILL1168, pILL1218) producing the AggB and AfaE-III proteins.

Table 3
Invasion of HeLa cells by *E. coli* HB101 producing AfaD or *AggB*

| Strain | Production of: | | Relative invasion (%)[a] |
|---|---|---|---|
| | Invasin | Adhesin | |
| HB101(pILL1101) | AfaD-III | AfaE-III | 100 |
| HB101(pILL1168) | – | AfaE-III | 25.4 |
| HB101(pILL1168, pILL1218) | AggB | AfaE-III | 59.5 |
| HB101(pILL1218) | AggB | – | $<0.5$ |

[a]Invasion relative to that of *E. coli* HB101(pILL1101), which was defined as 100%.

the AggB protein in bacterial internalization by functional complementation assays. The recombinant plasmid pILL1218, which carries the *agg* gene cluster from the EAggEC 17-2 strain, contains a mutation in the pilin-encoding *aggA* gene that completely abolishes bacterial adhesion to epithelial cells. This plasmid was introduced into the AfaD-negative mutant, HB101(pILL1168), which contains the *afa-3* gene cluster from strain A30 with a mutated *afaD* gene and produces only AfaE-III [8]. We have previously reported [9], and we show here (Table 3), that HB101(pILL1168) has only 20% of the capacity of HB101 (pILL1101), which produces both AfaE-III and AfaD-III [9], to invade HeLa cells. When both pILL1218 and pILL1168 were present in a given strain, complementation occurred: the recombinant HB101-(pILL1218, pILL1168) showed a level of invasion more than twice that of HB101(pILL1168). Electron microscopy showed internalized HB101(pILL1218, pILL1168) bacteria (Fig. 3C). These data indicate that, like the AfaD products, the AggB protein mediates the internalization of adherent bacteria into epithelial cells.

## 4. Conclusions

We have previously shown that the *afa-3* gene cluster is unique among bacteria in that it encodes proteins involved in both bacterial adhesion to and invasion of HeLa cells [9]. In this work, we found that the *afaD* genes are structurally conserved among human *afa*-expressing *E. coli* isolates. Indeed, no AfaD subtype was associated with a particular AfaE adhesin subtype (i.e. with afimbrial or fimbrial morphology) or with a specific disease (UTI or diarrhea). The various recombinant AfaD proteins promoted the entry of beads into HeLa cells and *afaD*-expressing bacteria producing different AfaE subtypes invaded HeLa cells. This suggests that AfaD proteins are also conserved at the functional level. We have recently reported that, although they are only 45% identical to the AfaD-III of A30, the AfaD-like proteins AfaD-VII and AfaD-VIII, encoded by *afa*-like operons from bovine pathogenic *E. coli*, also mediate bacterial internalization [7]. Here, we demonstrate that, like the AfaD-VII and AfaD-VIII proteins, the AggB product encoded by the aggregative adhesion fimbria I gene cluster from EAggEC isolates, is an invasin. Interestingly, *agg*-expressing EAggEC strains may be taken up by HeLa cells [22]. It is unknown whether invasion is specifically promoted by AggB in these clinical strains. The AafB protein product, which is encoded by the aggregative adhesion fimbria II gene cluster from EAggEC strains, and is similar to both AfaD-III and AggB (63.9 and 64.8% similarity, respectively), is probably also an invasin. Both *agg*-expressing EAggEC and *afa*-expressing diffusely-adherent *E. coli* strains are enteric pathogens and have been associated with persistent diarrhea [1,2,6,14,21]. For such isolates, the ability to invade epithelial cells may be an evolved strategy for persistence within the host, preventing a deleterious interaction with host defense barriers. This notion is strongly supported by the recent report of Edwards et al. [23] concerning the in vivo role of SefD, a protein produced by another enteric pathogen, *Salmonella enteritidis*. SefD, encoded by the *sef* adhesion system, has been demonstrated to be absolutely required for the internalization of bacteria by peritoneal macrophages and for full virulence in vivo of *S. enteritidis* [23,24]. AfaD-like invasins and SefD, which is 21.6% identical (29.2% similar) to AfaD-III, may have a common ancestor.

All the known AfaD-like invasins are encoded by gene clusters that also encode adhesins. The adhesin plays a crucial role in the interaction of *afa*-expressing strains with HeLa cells [9]. Despite their structural variability, the AfaE adhesins produced by human *afa*-expressing *E. coli* isolates are functionally similar, promoting recruitment of the receptor molecule, DAF, on epithelial cells [25,26]. However, the AfaE-VII and AfaD-VIII adhesins (produced by bovine-pathogenic *E. coli*) and the AggA pilin (produced by human EAggEC strains) do not recognize the human DAF as a receptor or promote its recruitment ([7]; unpublished data). Invasion mediated by AfaD is absolutely dependent on the initial adhesion step. AfaE adhesins or AggA pilins may therefore recruit their receptors (DAF or other molecules) locally and the resulting tight bacterium–epithelial cell interaction may increase the efficiency of recruitment of the invasin receptor for subsequent bacterial internalization [9]. As previously shown for AfaD-VII and AfaD-VIII, we observed in this study that the AggB invasin complemented an AfaD-negative mutant, indicating that DAF capping induced by AfaE adhesins can recruit AfaD-VII-AfaD-VIII or AggB-receptor molecules to promote invasion. Therefore, it is possible that all these invasins use the same or related receptors, but not DAF as shown by experiments with CHO cells. Identification of the specific cell receptors for AfaD-like invasins should increase our understanding of the internalization process and of the role of invasion in the pathophysiology of infections caused by *E. coli* strains expressing gene clusters, such as the *afa* clusters, encoding proteins for both adhesion to and invasion of epithelial cells.

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