The secreted autotransporter toxin, Sat, functions as a virulence factor in Afa/Dr diffusely adhering Escherichia coli by promoting lesions in tight junction of polarized epithelial cells

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Summary
Afa/Dr diffusely adhering Escherichia coli (DAEC) strains are responsible for urinary tract and intestinal infections. Both in intestine and kidney, the epithelial cells forming epithelium are sealed by junctional domains. We provide evidence that the Secreted autotransporter toxin, Sat, belonging to the subfamily of serine protease autotransporters of Enterobacteriaceae (SPATEs), acts as a virulence factor in Afa/Dr DAEC by promoting lesions in the tight junctions (TJs) of polarized epithelial Caco-2/TC7 cells. Southern blot analysis reveals that the prototype strains of the subclass-1 and subclass-2 typical Afa/Dr DAEC strains, hybridize with a sat probe. Using the wild-type IH11228 strain, the recombinant E. coli AAEC185 strain that expresses Sat, and other strains expressing Dr adhesin and Sat, we report that Sat induces rearrangements of the TJs-associated proteins ZO-1, ZO-3 and occludin, and increases the formation of domes as the result of an increase in paracellular permeability without affecting the transepithelial electrical resistance of the cell monolayers. Moreover, we observe that Sat-induced disassembly of TJs-associated proteins is dependent on the serine protease motif. Finally, an analysis of the prevalence of the sat gene in three collections of Afa/Dr DAEC strains collected from the stools of children with and without diarrhoea, and from the urine of patients with urinary tract infection (UTI) shows that:

(i) the sat gene is highly prevalent in UTI-associated Afa/Dr DAEC strains (88% positive), (ii) the sat gene is generally absent from Afa/Dr DAEC strains collected from the stools of children without diarrhoea (16% positive); whereas (iii) it is present in about half of the strains collected from the stools of children with diarrhoea (46% positive).

Introduction
Among human pathogenic Escherichia coli (Kaper et al., 2004), the diffusely adhering E. coli (DAEC) strains are responsible for recurrent urinary tract and gastrointestinal infections. DAEC strains appear to form a heterogeneous group of virulent E. coli. It has recently been proposed that the DAEC should be divided into two classes of strains, the typical DAEC and the atypical DAEC, each class being further subdivided into two subclasses (Servin, 2005). The class of typical DAEC includes E. coli strains that harbour Afa/Dr adhesins (Afa/DrDAF) that: (i) have the same genetic organization, (ii) are able to bind to human decay accelerating factor (DAF, CD55) and (iii) promote DAF mobilization. This class includes two subclasses of strains, subclass-1 of typical adhesins, that bind to the human carcinoembryonic antigen (CEA, CD66e) (Afa/DrCEA), and subclass-2 of typical adhesins, that do not bind to human CEA. The typical class of Afa/Dr DAEC expresses a family of genes that is organized in an operon and that all share a similar genetic organization, consisting of at least five genes (A to E), of which the last gene, E, encodes the major structural adhesins that act as virulence factor. These adhesins include AfaE-I (Labigne-Roussel et al., 1984), AfaE-III (Le Bouguenec et al., 1993), Dr (Nowicki et al., 1987), Dr-II (Pham et al., 1997), F1845 (Bilge et al., 1989) and NFA-I (Ahrens et al., 1993). In epithelial cells, the membrane-associated glycosylphosphatidylinositol (GPI)-anchored proteins DAF (CD55), CEA (CD66e) and CEACAM6 (non-specific cross-reacting antigen, NCA, CD66c), and the transmembrane anchored CEACAM1 (biliary glycoprotein, BGP, CD66a) act as receptors for the typical Afa/Dr DAEC adhesins. Once a cell has been infected by Afa/Dr DAEC, many subsequent cellular events are dependent on the...
Our group had previously reported that wild-type Afa/Dr DAEC strain C1845 promotes lesions in the junctional domain of cultured human intestinal, monolayer-forming Caco-2 cells that are characterized by an increase in the paracellular permeability without any reduction of the transepithelial resistance (TER) of the monolayers, and by rearrangements in the distribution of tight junctions (TJs)-associated occludin and ZO-1 proteins (Peiffer et al., 2000a). Interestingly, the induced cell injuries develop independently of the C1845 adhesin suggesting that at least one other pathogenic factor, distinct from the C1845 adhesin, also play(s) a role in Afa/Dr DAEC pathogenesis. Taddei et al. (2003) have recently demonstrated the prevalence of the sat gene that encodes for the secreted autotransporter toxin, Sat, in DAEC strains collected from the stools of children with and without diarrhoea, and from urine cultures. Sat belongs to the subfamily of serine protease autotransporters of Enterobacteriaceae (SPATEs) that are the predominating secreted proteins in several pathogens (Henderson and Nataro, 2001). The sat gene has been previously identified in the CFT073 strain (Guyer et al., 2000), a prototype of a uropathogenic strain of E. coli (UPEC) (Mobley et al., 1990), where it resides within the pathogenicity island I (PAI-IcFT073) (Welch et al., 2002). Our group has previously reported that the uropathogenic Afa/Dr DAEC strain IH11128 and the diarrhoea-associated Afa/Dr DAEC strain C1845 harbour part of the pathogenicity-associated island (PAI-IcFT073) (Guignot et al., 2000a; Blanc-Potard et al., 2002).

We show that the sat gene was present in the prototype strains of the typical class of Afa/Dr DAEC (Afa/DrDAEC), including E. coli KS52 (AfaE-I), A30 (AfaE-III), IH11128 (Dr), EC7372 (Dr-II), C1845 (F1845) and 827 (NFA-I). We have conducted a set of experiments to investigate the role of Sat in Afa/Dr DAEC pathogenesis. We show that the wild-type Afa/Dr DAEC strain IH11128 produces a functional Sat, and also demonstrate that the toxin was responsible for the Afa/Dr adhesin-independent lesions in the junctional domain of polarized epithelial Caco-2/TC7 monolayer-forming intestinal cells.

### Results

**The sat gene is present in prototype Afa/Dr DAEC strains expressing the typical subclass-1 and subclass-2 Afa/Dr adhesins**

We analyse the presence of the sat gene by Southern blot with a probe specific for sat generated by PCR, corresponding to 744 bp of sat from CFT073. The wild-type Afa/Dr DAEC strains KS52 (AfaE-I), A30 (AfaE-III), AL851 (AfaE-V), IH11128 (Dr), EC7372 (Dr-II), C1845 (F1845) and 827 (NFA-I) are typical subclass-1 and subclass-2 of Afa/Dr DAEC (Servin, 2005). As reported in Table 1, all these strains mobilized DAF and hybridized with the sat probe. In contrast, the wild-type bovine 239KH89 (AfaE-VII) and 262KH89 (AfaE-VIII) strains failed to mobilize DAF and to hybridize with the sat probe.

**Chromosomal location of the sat gene in the wild-type Afa/Dr DAEC strain IH11128**

We decided to use one prototype strain of Afa/Dr DAEC, the strain IH11128, in order to get a clearer picture of the sat gene from Afa/Dr DAEC and of its role in Afa/Dr DAEC pathogenesis. The sat gene from strain IH11128 (satIH11128) was amplified by PCR using specific primers defined according to the sequence of Sat from CFT073 (Guyer et al., 2000; accession number AF289092). The PCR fragment of the expected size was amplified and cloned into pACYC184. Sequencing of the sat gene from strain IH11128 compared with sat gene from strain CFT073 showed 15 mutated nucleotides (99.6% identity), resulting in only five mutated amino acids (R140K, S579T, Y612H, V669A and I104M).

To determine where the satIH11128 gene is located on the chromosome, we used the thermal asymmetric interlaced (TAIL)-PCR method to amplify the 3′ and 5′-flanking

### Table 1. Distribution of the sat gene in prototype Afa/Dr DAEC strains and Afa/Dr-related strains.

| Strains | Origin   | DAF mobilization | sat |
|---------|----------|------------------|-----|
| KS52 (AfaE-I) | UTI human | + | + |
| A30 (AfaE-III) | UTI human | + | + |
| AL851 (AfaE-V) | UTI human | + | + |
| 239KH89 (AfaE-VII) | Diarrhoea calf | - | - |
| 262KH89 (AfaE-VIII) | Diarrhoea calf | - | - |
| IH11128 (Dr) | UTI human | + | + |
| 7372 (Dr-II) | UTI human | + | + |
| C1845 (F1845) | Diarrhoea human | + | + |
| 827 (NFA-I) | UTI human | + | + |

*a. In parentheses, adhesins.
b. Mobilization of DAF around bacteria adhering to CHO cells transfected with DAF cDNA, detected using a DAF monoclonal antibody 8D11.
c. Positive for the sat probe described in Experimental procedures.*

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region of sat_{IH11128}. PCR fragments were cloned into TOPO-XL vectors and then sequenced. We found that the 5′-flanking region of sat_{IH11128} was very similar to the 5′-flanking region of sat_{CFT073} gene over more than 1 kb, corresponding to PAI-I_{CFT073} (with the iutA gene). However, on the 3′-flanking region of sat_{IH11128} gene, the first 200 bp was nearly identical that the sequence found in CFT073 (99.9%), but completely different thereafter. A sequence homologous to IS600 was also found. This finding suggests that sat_{IH11128} gene is likely to be located on the PAI that had already been reported in IH11128, but that this island is significantly different from PAI-I_{CFT073} (Blanc-Potard et al., 2002).

Identification of the Sat protein secreted by the wild-type strain IH11128

The culture supernatants of the wild-type strains CFT073 and IH11128 were concentrated; the proteins were denatured and electrophoresed on an SDS 10%polyacrylamide gel (Fig. 1). The silver-stained SDS-PAGE analysis in Fig. 1 shows that at least three bands were localized within the 100–130 kDa region for the strain CFT073, accordingly that this strain expresses at least four toxins with molecular masses ranging between 100 and 130 kDa. For strain IH11128, a single band was localized around 100–130 kDa. After excision of this band, a MALDI-TOF (matrix-associated laser desorption ionization time of flight) mass spectrometric analysis was conducted (measured peptides: 85; matched peptides: 12; minimum sequence coverage: 11; score 57). Comparative analysis using the MASCOT peptide mass fingerprint software shows that the protein corresponds to Sat (NCBI accession number: AF289092) (not shown). Similar results were obtained using supernatants of cultures of recombinant E. coli strains expressing sat genes from IH11128 (psat_{IH11128}) or CFT073 (psat_{CFT073}).

Effect of Sat on TJs gate functions

Caco-2 cells are known to form domes on plastic supports under standard culture conditions resulting of an accumulation of fluids at the basolateral domain of the cells forming monolayers (Grasset et al., 1984). Observation and counting of domes were conducted by interferential phase contrast light microscopic examination of Caco-2/TC7 monolayers infected with the non-pathogenic E. coli strain AAE185, recombinant E. coli strain that expresses Sat (psat_{IH11128}), or recombinant E. coli strain that expresses both Dr adhesin and Sat (Dr/psat_{IH11128}). As shown in Fig. 2A, in cells infected with recombinant E. coli expressing Sat (psat_{IH11128}) or recombinant E. coli strain expressing both Dr adhesin and Sat (Dr/psat_{IH11128}), the numbers of domes increased as compared with cell monolayers infected with the non-pathogenic E. coli AAE185.

Domes formed in Caco-2 cell monolayers result from fluid accumulates in randomly distributed areas that evolve permanently into the monolayers (Grasset et al., 1985). Our results showing that the number of fluid-formed domes increases following infection with recombinant E. coli expressing Sat suggest that the paracellular permeability of infected Caco-2/TC7 cell monolayers is increased. In order to demonstrate this effect, the paracellular diffusion of non-ionic molecular tracers was determined. Paracellular permeability was measured using defined paracellular markers having different sizes: \textsuperscript{14}C-mannitol (182 Da) and fluorescein-5 sulphonic acid (FSA) (478 Da). The mucosal-to-serosal flux rate of markers across the filter-grown Caco-2/TC7 monolayers was determined at 5 h post infection (Fig. 2B and C). The rate of unidirectional flux of markers was negligibly low in control monolayers (not shown). Infection of Caco-2/TC7 cell monolayers with recombinant E. coli expressing Sat (psat_{IH11128}) or recombinant E. coli strain expressing both
Dr adhesin and Sat (Dr/pSATIH11128) resulted in a highly significant increase in the paracellular permeability for 14C-mannitol as compared with monolayers infected with the non-pathogenic E. coli AAEC185 (Fig. 2B). In contrast, no change in the paracellular permeability of FSA was found in the infected cell monolayers (Fig. 2C).

Whether change in transepithelial electrical resistance (TER) develops was investigated in infected Caco-2/TC7 cell monolayers. As compared with filter-grown Caco-2/TC7 monolayers infected with the E. coli AAEC185 strain, no change in TER was found in cell monolayers infected with the recombinant E. coli expressing Sat (pSATIH11128) or recombinant E. coli strain expressing both Dr adhesin and Sat (Dr/pSATIH11128) (Fig. 2D). We have controlled the response of the Caco-2/TC7 monolayers using the enteropathogenic E. coli (EPEC) wild-type strain E2348/69. Caco-2/TC7 cell monolayers infected with E2348/69 bacteria show the typical EPEC-induced dramatic decrease of TER (Philpott et al., 1996) (Fig. 2D).

**Sat induces rearrangements in TJs-associated proteins in Caco-2/TC7 cells**

In polarized, epithelial, intestinal, monolayer-forming cells, the most apical structure of the junctional complex is the TJ that acts as the major paracellular barrier (Matter and Balda, 2003). Structural proteins have been identified as being specifically associated with the TJs, including several cytoplasmic protein members of the membrane-associated guanylate kinase (MAGuK) family of proteins comprising the zonula occludens-1, -2, -3 and -4 proteins (ZO-1, -2, -3 and -4). In addition, the functional transmembrane proteins occludin and claudins are localized to TJs strands. Our group has previously...
reported that adhesin-independent lesions in TJs follow the infection of polarized monolayer-forming Caco-2/TC7 cells by wild-type Afa/Dr DAEC strain C1845 (Peiffer et al., 2000a). We investigated whether Sat induces rearrangements in the distribution of ZO-1, ZO-3, occludin and claudin-1 in Caco-2/TC7 cells (Fig. 3). For this purpose, the cells were infected with a non-pathogenic E. coli strain AAEC185, wild-type strain IH11128, recombinant E. coli strain that expresses Dr adhesin (DrIH11128), recombinant E. coli strain that expresses Sat (pSATIH11128), or recombinant E. coli strain that expresses both Dr adhesin and Sat (Dr/pSATIH11128).
Using confocal laser scanning microscopy (CLSM) analysis, the labelling of the ZO-1, ZO-3, occludin and claudin-1 in Fig. 3 shows that all the proteins were normally localized at sites of cell–cell boundaries in cells infected with the non-pathogenic E. coli strain AAEC185, and that the cells formed the typical sharp, honeycomb-like organization of cultured intestinal cells at subconfluency. The distribution of ZO-1, ZO-3 and occludin was characterized by a brightly stained continuous band lining each cell, whereas the band staining for claudin-1 was larger. Following the infection of Caco-2/TC7 cells by the wild-type strain IH11128, the distributions of ZO-1, ZO-3 and occludin were profoundly modified, and characterized by a reduction of the staining at the cell–cell boundaries or by a marked break in the brightly stained band indicating the loss of the proteins from the TJs (Fig. 3). Distribution of claudin-1 in IH11128-infected Caco-2/TC7 cells appears modified at a lesser extent as compared with ZO-1, ZO-3 and occludin. Consistent with a previous report (Peiffer et al., 2000a), immunolabelling of TJs-associated proteins is not modified in cells infected with the recombinant E. coli DrIH11128 (Fig. 3). When infected by the recombinant E. coli psaIH11128, the changes in the distribution of ZO-1, ZO-3 and occludin mirrored the changes observed in the cells infected with the wild-type strain IH11128 (Fig. 3). Although CLSM analysis may be of limited quantitative accuracy, the rearrangements in TJs-associated proteins were more pronounced in cells infected with the recombinant E. coli DrpsaIH11128 expressing both Sat and Dr adhesin (Fig. 3). This finding suggests that the delivery of the Sat at the vicinity of the cell membrane could allow more efficient binding of Sat to its unknown receptor, a step necessary for optimal toxin activity.

In order to get better insight on TJs injuries promoted by Sat, the expression of ZO-1, ZO-3, occludin and claudin-1 was examined by Western blot analysis in Caco-2/TC7 cells infected with the non-pathogenic E. coli strain AAEC185, wild-type strain IH11128, recombinant E. coli strain that expresses Sat (psaIH11128), or recombinant E. coli strain that expresses both Dr adhesin and Sat (DrpsaIH11128) (Figs 4 and 5). In the total cell extracts of cells infected with the non-pathogenic E. coli strain AAEC185, ZO-1, ZO-3 and claudin-1 were expressed as a single band and occludin was expressed as two bands corresponding to the non-phosphorylated and hyperphosphorylated forms of the protein (Fig. 4A). Quantification by scanning of the bands shows that the expression of ZO-1, ZO-3 and in a lesser extent of occludin was decreased in cells infected with wild-type IH11128, recombinant E. coli psaIH11128 or recombinant E. coli DrpsaIH11128 as compared with cells infected with the non-pathogenic E. coli strain AAEC185 (Fig. 4B). In contrast, expression of claudin-1 was not modified (Fig. 4B). We next investigated by Western blot analysis the expression of ZO-1, ZO-3, occludin and claudin-1 in Triton-X100-soluble and Triton-X100-insoluble fractions (Fig. 5). Quantification by scanning of the bands shows that the expression of ZO-3 and occludin is highly decreased in both Triton-X100-soluble and Triton-X100-insoluble fractions of psaIH11128- and DrpsaIH11128-infected cells as compared with cells infected with the non-pathogenic E. coli strain AAEC185. In contrast, there is an increase of the claudin-1 expression in Triton-X100-soluble fractions of cells infected with recombinant E. coli psaIH11128, and DrpsaIH11128 as compared with cells infected with the non-pathogenic E. coli strain AAEC185.

Altogether, the results obtained by CLSM analysis and by Western blot analysis demonstrate that Sat is sufficient to promote rearrangements in TJs-associated proteins.

Serine protease activity is necessary for Sat-induced lesions in TJs

Sat contains a serine protease motif that plays a pivotal role in the activity of SPATEs, and plasmid-encoded toxin (Pet) and EspC have been shown to act via their conserved serine protease motif (GDSDSG) (Dutta et al., 2002). To test the role played by protease activity in Sat-induced TJs lesions, we used the protease inhibitor phenylmethylsulphonyl fluoride (PMSF). Navarro-Garcia et al. (1999) have reported that a mutation in the catalytic serine residue of the toxin Pet abolishes the protease activity, cytopathic effects, cytoskeletal damage, and enterotoxigenic effects. To confirm the role of serine protease activity in Sat-induced TJs lesions, a site-directed mutation was performed. A mutant has been constructed in which an isoleucine replaces the serine at residue 256 of the serine protease motif of Sat (psaIH11128S256I). As can be seen in Fig. 6A, the Caco-2/TC7 cells infected with the wild-type IH11128 bacteria or with recombinant E. coli
Sat does not induce dissociation of the F-actin network in Caco-2/TC7 cells

The actin cytoskeleton plays a crucial role in the regulation of TJs by mechanisms that involve the regulation of cortical actin contraction and direct interactions between the actin cytoskeleton and certain TJ components. Two SPATEs, Pet and EspC, produce the loss of F-actin stress fibres (Navarro-Garcia *et al.*, 1999; 2004). Our group has previously reported that infection of enterocyte-like Caco-2 cells by Afa/Dr DAEC is followed by a dramatic disorganization of the brush border-associated F-actin (Bernet-Camard *et al.*, 1996; Peiffer *et al.*, 2000b). We decided to investigate whether Sat is involved in this phenomenon. For this purpose, we used CLSM analysis to examine the organization of the F-actin network in Caco-2/TC7 cells infected with the wild-type strain IH11128 or the recombinant *E. coli* strain expressing Sat alone (psat*IH11128*). In agreement with previous reports (Bernet-Camard *et al.*, 1996; Peiffer *et al.*, 2000b), the cells infected with the wild-type strain IH11128 or the recombinant *E. coli* strain expressing Sat alone (psat*IH11128*) have a normal distribution of the ZO-1 protein (Fig. 6B).

psat*IH11128* and treated with PMSF showed a restored distribution of ZO-1 protein that resembles the distribution of the protein in cells infected with the non-pathogenic *E. coli* strain AAEC185. Moreover, Caco-2/TC7 cells infected with the recombinant *E. coli* psat*IH11128*S256I have a normal distribution of the ZO-1 protein (Fig. 6B).

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Prevalence of the sat gene in Afa/Dr DAEC isolates

Epidemiological studies have shown that Afa/Dr DAEC strains are involved in 25–50% of cases of cystitis in children and 30% of cases of pyelonephritis in pregnant women, and in diarrhoea in infant (Servin, 2005). We examined whether the sat gene is present in Afa/Dr DAEC clinical isolates from urinary tract infections (UTIs),
from non-diarrhoeagenic and diarrhoeagenic sources (Table 2). The strains were examined: (i) for the mobilization of DAF receptor using CHO cells stably transfected with cDNA encoding human DAF and mAb directed against DAF and (ii) for the presence of the sat gene by Southern blot, using the sat probe described above. Among the 136 Afa/Dr DAEC strains examined, 106 strains (76.3%) displayed mobilization of DAF on infected CHO cells stably transfected with DAF cDNA. Among the 106 Afa/Dr DAEC strains displaying DAF mobilization, 52 strains (49%) hybridized with the sat probe. Our findings reveal the high prevalence of the sat gene in UTI-associated Afa/Dr strains. Indeed, 29 of the 33 Afa/Dr DAEC strains associated with UTI hybridized with the sat probe (88%). In the group of 37 Afa/Dr DAEC strains not associated with diarrhoea only 16 strains hybridized with the sat probe (46%). Interestingly, in the group of 35 Afa/Dr strains associated with diarrhoea, 16 strains (46%) hybridized with the sat probe. Moreover, we found that the 19 strains associated with diarrhoea and that are negative for sat did not hybridize with a probe generated by PCR, corresponding to 669 bp of the conserved C-terminal autotransporter region of SPATEs, suggesting that these strains do not carry other SPATEs genes.

**Discussion**

Afa/Dr DAEC strains are responsible for urinary tract and intestinal infections (Servin, 2005). Both in intestine (Louvard et al., 1992) and kidney (Gonzalez-Mariscal et al., 2000), the epithelia are joined to each other via a set of intercellular junctions. Among the well-identified intercellular junctions, TJ plays a particular role by sealing adjacent epithelial cells just beneath their apical surface. Moreover, TJ is the intercellular junction that regulates diffusion between cells and therefore allows the epithelium to form cellular barriers that separate compartments with differing compositions (Matter and Balda, 2003). The intercellular gate formed by TJs is not only highly regu-
Table 2. Presence of the sat gene in UTI-, non-diarrhoea-, and diarrhoea-associated E. coli isolates that tested positive for afa1-afa2 or daaC probes.

| Origin                  | Number of strains | DAF mobilizationa | DAF mobilization and sat positive | DAF mobilization and sat negative |
|-------------------------|-------------------|-------------------|-----------------------------------|----------------------------------|
| Total strains           | 136               | 106 (76.3)1       | 52 (49)2                          | 54 (51)2                          |
| UTIb                    | 47               | 33 (70)1          | 29 (88)2                          | 4 (12)2                           |
| Without diarrhoea        | 41               | 37 (90)1          | 6 (16)2                           | 31 (84)2                          |
| Diarrhoea               | 48               | 35 (73)1          | 16 (46)2                          | 19 (54)2                          |

a. Mobilization of DAF around bacteria adhering to CHO cells stably transfected with cDNA encoding DAF (Goluszko et al., 2001), detected using the DAF monoclonal antibody 8D11.
b. Strains isolated from urine specimens from patients (children and adults) with clinically diagnosed UTIs (Le Bouguenec et al., 2001).
c. Strains positive for the afa1-afa2 PCR (Le Bouguenec et al., 2001).
d. Strains isolated from stool specimens from children in Brazil and France with or without diarrhoea (Jallat et al., 1993; 1994; Forestier et al., 1996; Scaletsky et al., 2002).
e. Strains positive for the daaC probe described by Scaletsky et al. (2002).
f. Strains positive for the daaC probe described by Jallat et al. (1993).

In parentheses: % of strains examined and % of strains showing DAF mobilization.

lated, but is size- and ion-selective and therefore constitutes a semipermeable diffusion barrier that forms a morphological and functional boundary between the apical and basolateral cell surface domains. They also contribute directly to maintaining cell surface polarity by forming a fence that prevents the apical/basolateral diffusion of lipids and proteins. Whereas in the different parts of the intestine TJs were homogeneously present, they are non-homogeneously expressed in the different regions of kidney with a weak expression along the periphery of proximal tubules cells, and an increased expression along the ascending and descending loop of Henle, and in the distal region. Cross-talk between pathogenic bacteria and epithelial TJs can be mediated by a variety of virulence factors, consisting mainly of secreted toxins, or can be induced by direct contact between the pathogen and the epithelial membrane, followed by the injection into the cell of bacterial effectors (Hofman, 2003). Our group had previously reported that Afa/Dr DAEC strains promote lesions in the TJs of Caco-2/TC7 cell monolayers (Peiffer et al., 2000a). Bacteria and/or toxins can trigger actin cytoskeleton reorganization, activation of cellular signal transduction involved in the regulation of TJs, cleavage or modification of TJs-associated proteins, or rearrangements of TJs-associated protein organization (Hofman, 2003). Here, we provide the first evidence that Sat is responsible of the Afa/Dr DAEC-induced lesions in the TJs of fully polarized intestinal monolayer-forming Caco-2 cells by inducing rearrangements of the TJs-associated proteins ZO-1, ZO-3, occludin and claudin-1.

Sat is responsible for the Afa/Dr DAEC-induced lesions in the TJs of polarized intestinal monolayer-forming cells

The role of SPATEs in lesions promoted within the TJs of intestinal cells forming monolayers is not currently documented. Peiffer et al. (2000a) have reported that infection of Caco-2/TC7 monolayers with Afa/Dr DAEC wild-type strain C1845 Afa/Dr DAEC is accompanied by an increase in the paracellular permeability for mannitol and by an increase in the number of domes. In a monolayer of Caco-2 cells, the domes formed result from fluid accumulates (Grasset et al., 1984) due to an active paracellular vectorial ion transport as it has been revealed by confocal microscopy in cells forming domes (Rotoli et al., 2002). Here, we found that Sat is responsible for the increase in the number of domes formed in Afa/Dr DAEC-infected Caco-2/TC7 cell monolayers. A similar increase in domes formation has been observed with the Helicobacter pylori VacA toxin which induces an increase of mannitol and sucrose transmonolayer flux together with an alteration in distribution of ZO-1 and occludin (Papini et al., 1998). In addition, we demonstrate that Sat increases the paracellular passage of mannitol with no change in the passage of a non-ionic molecule having a higher molecular mass. The observed increase in Sat-induced formation of domes which results from an increase of the paracellular passage of fluid at the basolateral domain of the Caco-2/TC7 cell monolayers and observation by Taddei et al. (2005) that Sat induces marked fluid accumulation in rabbit ileum loops, are indicating that Afa/Dr DAEC strains expressing Sat may be involved in diarrhoea.

It has been previously observed that the increase in paracellular permeability in Caco-2/TC7 cell monolayers infected with the wild-type Afa/Dr DAEC strain C1845 develops without affecting the TER (Peiffer et al., 2000a). Here we found the same phenomenon since in Caco-2/TC7 cell monolayers infected with the wild-type Afa/Dr DAEC strain IH11128 or the recombinant E. coli expressing Sat, the increase in paracellular permeability and the rearrangements in TJs-associated proteins develop without TER modification. This could be explained by the
fact that Sat affects differently the distribution and expression of TJs-associated proteins. Indeed, results show that Sat highly decreased the expression of ZO-1, ZO-3 and to a lesser extent of occludin both in the total cell extracts, Triton-X100-soluble and Triton-X100-insoluble fractions. In contrast, Sat little affects the distribution of claudin-1 at the TJs, but does not modify the expression of claudin-1 in total cells extracts. Claudin-1 (Furuse et al., 1998) belongs to the claudin superfamily consisting of at least 18 homologous proteins in humans that are important structural and functional components of TJs in paracellular transport (Heiskala et al., 2001). These proteins complexed with two other integral transmembrane proteins, occludin and junctional adhesion molecule and interacted directly with ZO-1, ZO-2 and ZO-3. Interestingly, among TJs-associated proteins claudins are believed to be more important in regulating the resistance of the TJs-dependent electrical seal in the monolayers. Indeed, it has been reported that claudin-1-overexpressing Madin Darby Canine Kidney (MDCK) cells exhibited about four times higher TER than wild-type MDCK cells (Inai et al., 1999). To explain whether the TER is not decreased in Sat-exposed Caco-2/TC7 cells, an hypothesis not exclusive of other is that the non-modified expression of claudin-1 is sufficient to maintain a normal TER in the toxin-treated cells.

Several bacterial toxins act on TJs and by different mechanisms alter the distribution and/or the association of structural and functional TJs-associated proteins (Hofman, 2003). For example, Clostridium difficile toxin A decreases TER and increases paracellular permeability by mechanisms involving RhoA glucosylation and protein kinase C signalling that in turn lead of actin depolymerization, translocation of ZO-1 and ZO-2, and dephosphorylation or translocation of occludin (Nusrat et al., 2001; Chen et al., 2002). Clostridium perfringens enterotoxin has been shown to bind to claudin-3 and -4, but not to claudin-1 or -2 (Fujita et al., 2000), and removes claudin-3 and -4 from TJs strands (Sonoda et al., 1999). Moreover, proteolytic activity of bacterial toxins against junctional proteins has been observed. Bacteriodes fragilis toxin, flaglysin cleaves the E-cadherin generating two breakdown products of 28 and 33 kDa (Wu et al., 1998). Moreover, the Vibrio cholerae zinc binding metalloprotease, haemagglutinin/protease degrades occludin into two breakdown products of 50 kDa and 35 kDa (Wu et al., 2000). It has been demonstrated that SPATEs induced the cleavage of numerous proteins (Henderson and Nataro, 2001; Dutta et al., 2002). For example, spectrin degradation and production of a 120 kDa subproduct has been observed with Pet (Villaseca et al., 2000). Moreover, Pet cleaves fodrin generating two breakdown products of 37 and 72 kDa (Canizalez-Roman and Navarro-Garcia, 2003). The mechanism(s) by which Sat induces the rearrangements in TJs-associated proteins remains to be determined. By Western blot analysis, our results are indicating that ZO-1, ZO-3 and occludin disappeared in Sat-exposed cells. An hypothesis is that Sat could exert a proteolytic activity against these TJs-associated proteins. Western blot analysis of ZO-1, ZO-3 and occludin in Sat-exposed cells is not in favour of this mechanism of action because results reveals no breakdown products. However, it should be of interest to conduct a more detailed analysis to examine whether or not Sat induces the cleavage of proteins regulating the organization of TJs-associated proteins.

Various SPATEs have been identified in enterovirulent E. coli, including the Pet of enteroaggregative E. coli (EAEC) (Eslava et al., 1998), the EspP (Bruder et al., 1997) and EpeA (Leyton et al., 2003) proteases of enterohaemorrhagic E. coli (EHEC), the Shiga toxin-producing E. coli (STEChalf et al., 1997), the EspC protein of EPEC (Stein et al., 1996; Mellies et al., 2001), the protein involved in intestinal colonization (Pic) of EAEC which is also found in Shigella (Henderson et al., 1999), the Pic protein of uropathogenic E. coli (PicU) which is homologous to the Pic protein of EAEC and Shigella (Parham et al., 2004), and the Era (ETEC autotransporter A) of enterotoxigenic E. coli (ETEChalf et al., 2004). Although Shigella does carry the sat gene (Ruz et al., 2002a; Niyogi et al., 2004), but the role of Sat in Shigella-induced TJs lesions has not been demonstrated. Interestingly, Sakaguchi et al. (2002) have observed that the Shigella-induced disruption of TJs is supported by secreted bacterial products. Whether the SPATEs EspC and EspP, produced by EPEC and EHEC, are involved in the TJs lesions produced by these enterovirulent E. coli (Philpott et al., 1996; 1998) remains to be determined.

Sat does not contribute to the Afa/Dr DAEC-induced cytoskeleton injuries in the apical domain of polarized intestinal cells

The actin cytoskeleton plays a crucial role in the regulation of TJs by mechanisms that involve the regulation of cortical actin contraction, and direct interactions between the actin cytoskeleton and certain components of TJs. Pet, EspP and EspC all promote the loss of F-actin stress fibres in HEp-2 and undifferentiated intestinal HT-29/C1 cells (Navarro-Garcia et al., 1999; 2004; Dutta et al., 2002). It has been previously reported that in cultured intestinal T84 cells infection with the EAEC strain 042 expressing a functional Pet toxin (Navarro-Garcia et al., 1999), damage to the apical membrane expressing a brush border is characterized by vesiculation and shedding of microvilli (Nataro et al., 1996). This phenomenon has also been found in Afa/Dr DAEC-infected Caco-2
cells (Bernet-Camard et al., 1996). Afa/Dr DAEC strains promote the destruction of the F-actin network in undifferentiated INT407 cells (Peiffer et al., 1998) and the disassembly of the brush border-associated proteins F-actin, villin and fimbrin in fully differentiated Caco-2 cells (Bernet-Camard et al., 1996; Peiffer et al., 2000b). Interestingly, Afa/Dr DAEC cell infection is followed by the disappearance of the spectrin labelling located centrally in the cells, and the appearance of randomly distributed small aggregates of clumped proteins (Peiffer et al., 2000b). Sat and Pet both cleaved spectrin, a heterodimeric cytoskeletal protein that serves to link F-actin filaments with other cytoskeletal proteins (Dutta et al., 2002; 2003). Our results demonstrate that despite the fact that Afa/Dr DAEC expresses Sat, this toxin is not involved in the Afa/Dr DAEC-induced alteration of the brush border-associated F-actin cytoskeleton in intestinal cells. Taken together, the foregoing results and those reported here suggest that the mechanisms of action of the SPATE toxins, Sat and Pet, in intestinal cells must either be different or the F-actin network must be differently regulated in the undifferentiated epithelial cells and differentiated intestinal cells.

**Role of Sat in Afa/Dr DAEC pathogenesis**

Urinary tract infections are among the most frequently acquired bacterial infections, and *E. coli* accounts for up to 90% of all UTIs. Risk factors for UTIs include host behaviour, host characteristics, and bacterial characteristics. The *sat* gene has been described to be present in 26–55% of UPEC strains, particularly in nalidixic acid-sensitive UPEC strains (Guyer et al., 2000; Ruiz et al., 2002b; Vila et al., 2002). Epidemiological studies have shown that Afa/Dr DAEC strains are involved in 25–50% of cases of cystitis in children, and 30% of cases of pylonephritis in pregnant women (Arthur et al., 1989; Le Bouguenec et al., 1992; Nowicki et al., 1994; Hart et al., 1996; 2001; Usein et al., 2001). Moreover, Dr adhesin-expressing *E. coli* has been shown to be associated with a doubling of the risk of a second UTI, suggesting its possible association with recurrent or chronic UTI (Zhang et al., 1997). The presence of virulence factors in uropathogenic Afa/Dr DAEC strains has previously been investigated (Guignot et al., 2000a). Here, tests to detect the *sat* gene in prototype strains of the subclass-1 and subclass-2 typical uropathogenic Afa/Dr DAEC strains (Servin, 2005) show that all these strains were *sat*-positive. Moreover, the analysis of the prevalence of the *sat* gene in a collection of Afa/Dr DAEC samples reveals a high prevalence of the *sat* gene in clinical isolates collected from the urine of patients with UTIs (88% positive). In the uropathogenic wild-type strain CFT073, the *sat* gene encoding Sat is located within PAI-I_CFT073 (Welch et al., 2002). The uropathogenic Afa/Dr DAEC strain IH11128 which expresses Dr adhesin and the diarrhoea-associated strain C1845 which expresses F1845 adhesin, harbour part of PAI-I_CFT073, but lack the central part of the island that carries the *hly* and *pap* operons (Blanc-Potard et al., 2002). We do not know whether the internal region of the PAI-I_CFT073 has simply been deleted or has been replaced by different genetic material in the Afa/Dr DAEC strains IH11128 and C1845. In our study, we show that *sat*IH11128 is located on this island, but that the 3′-end of *sat*IH11128 is flanked by an IS600-related sequence that is not present in PAI-I_CFT073. Interestingly, it has been shown recently that *E. coli* strain Nissle1917 also carries a PAI (GEI INissle1917, accession number: AJ586888) homologous to PAI-I_CFT073 that also carries *sat* (Grozdanov et al., 2004). As in IH11128, *sat* from GEI INissle1917 is flanked by an IS600-related sequence and lacks the central part of this island where the *hly* and *pap* operons are located. This suggests that PAI from Afa/Dr DAEC is likely to be more closely related to GEI INissle1917 than to PAI-I_CFT073, although it remains to be confirmed whether the *hly* and *pap* operons have been replaced by open reading frame encoding mobility factors, as in GEI INissle1917.

The involvement of Afa/Dr DAEC in diarrhoea is controversial. However, epidemiological studies have demonstrated an age-related incidence for Afa/Dr DAEC in diarrhoea in children (Baqui et al., 1992; Gunzburg et al., 1993; Levine et al., 1993; Scaletsky et al., 2002). Here, we have analysed the presence of the *sat* gene in two intercontinental collections of Afa/Dr DAEC collected from the stools of children with or without diarrhoea. We found that the *sat* gene was generally absent from Afa/Dr DAEC isolates collected from the stools of children without diarrhoea (16% positive) and was present in about half of the clinical isolates of Afa/Dr DAEC collected from the stools of children with diarrhoea (46% positive). The *sat* gene has been found in 44% of intestinal DAEC isolates (Taddei et al., 2003; 2005) and in *Shigella* isolates of differing geographical origins (55% positive in Africa and Asia, and 71% positive in Latin America) (Ruiz et al., 2002a). Observation here that Afa/Dr DAEC strains carrying the *sat* gene are responsible for lesions at the junctional barrier in Caco-2/TC7 monolayers suggests that Sat may play in part a role in Afa/Dr DAEC intestinal pathogenesis. The fact that 54% of the Afa/Dr DAEC clinical isolates isolated from stools of infants with diarrhoea do not carry the *sat* gene clearly indicates that other unknown virulence factor(s) function(s) in Afa/Dr DAEC strains for diarrhoea in infant. However, more rigorous epidemiological studies should be conducted in order to examine the presence of Sat in Afa/Dr DAEC strains associated with age-related diarrhoea in children.
Table 3. Bacterial strains used in this study.

| Strain          | Characteristics                                           | Reference                                      |
|-----------------|-----------------------------------------------------------|-----------------------------------------------|
| AAEC185         | *E. coli* K12 type 1 pili deleted (∆fimB−H)               | Blomfield et al. (1991)                       |
| IH11128         | Wild-type strain expressing Dr adhesin                    | Vaisaan-Rhen. (1984); Nowicki et al. (1987) |
| EC7372          | Wild-type haemolytic strain expressing Dr-II adhesin       | Pharm et al. (1997)                          |
| C1845           | Wild-type strain expressing F1845 adhesin                 | Bilge et al. (1989)                          |
| KSS2            | Wild-type strain expressing AfaE-I adhesin                 | Le Bouguenec et al. (1993)                   |
| A30             | Wild-type strain expressing AfaE-III adhesin               | Ahrens et al. (1993)                         |
| AL851           | Wild-type strain expressing AfaE-V adhesin                 | Zhang et al. (1997)                          |
| B27             | Wild-type strain expressing NFA-I adhesin                  | Ahrens et al. (1993)                         |
| CFT073          | Wild-type UPEC strain                                      | Molely et al. (1990)                         |
| E2348/69        | Wild-type EPEC strain                                      | Levine et al. (1985)                         |
| psat11128s256l  | Recombinant AAEC185 strain containing the plasmid that     | This study                                    |
|                 | encodes Sat, isolated from wild-type strain IH11128       |                                               |
| psatCF073       | Recombinant AAEC185 strain containing the plasmid that     | This study                                    |
|                 | encodes Sat, isolated from wild-type strain CFT073        |                                               |
| Dr sat11128     | Recombinant AAEC185 strain containing the plasmid that     | Berger et al. (2004)                         |
|                 | encodes Dr adhesin, isolated from wild-type strain IH11128|                                               |
| Dr/p sat11128   | Recombinant AAEC185 strain containing the plasmids that    | This study                                    |
|                 | encode Dr adhesin and Sat, isolated from wild-type strain  |                                               |
|                 | IH11128                                                    |                                               |

Experimental procedures

Reagents and antibodies

The rabbit polyclonal anti-ZO-1 (clone Z-R1), anti-ZO-3 (clone ZMD.261), anti-claudin-1 (clone MH25) and mouse monoclonal anti-occludin (clone OC-3F10) antibodies were from Zymed (Invitrogen, Cergy, France). Anti-DAF monoclonal antibody 8D11 was from D. Lublin (Department of Pathology, Washington University School of Medicine, St Louis, MI). Texas Red sulphonyl chloride (TRSC)-, cyanine 5 (Cy5)- and fluorescein isothiocyanate (FITC)-conjugated donkey anti-rabbit and anti-mouse were purchased from Jackson Immunoresearch Laboratories (West Grove, PA). FITC-phalloidin was from Molecular Probes (Eugene, OR). The 14C-mannitol (15–30 Ci mM−1) was from Amersham (Les Ulis, France). Fluorescein-5 sulphonic acid was from Molecular Probes (Eugene, OR).

Bacterial strains and growth conditions

The wild-type *E. coli* and recombinant strains used in this study and their origins are listed in Table 3. For this study, recombinant *E. coli* strains were obtained by transforming the *E. coli* AAEC185 strain, which lack type 1 pili (Blomfield et al., 1991).

Four previously partially characterized collections of human pathogenic *E. coli* strains that were positive for the daaC probe (Bilge et al., 1989) or positive for the afa1-afa2 PCR (Le Bouguenec et al., 2001), were used in this study. Forty-seven *E. coli* strains were isolated from urine specimens of patients (children and adults) clinically diagnosed with UTI (Le Bouguenec et al., 2001). Thirty-eight *E. coli* strains were isolated from stool specimens from children with diarrhoea and 21 *E. coli* strains were isolated from stool specimens from children without diarrhoea in France (Jallat et al., 1993; 1994) and Brazil (Scaletsky et al., 2002).

Stock cultures were maintained on 10% glycerol at −80°C. Before the experiments, the bacterial strains were transferred onto fresh Luria–Bertani (LB) agar (Difco Laboratories, Detroit, MI) and incubated at 37°C for 24 h. For each experiment bacteria were subcultured in LB broth at 37°C for 18 h. On the day of the experiment, bacteria were washed three times with sterile phosphate-buffered saline (PBS) and recovered using Dulbecco’s modified Eagle’s minimal essential medium (DMEM) (Invitrogen, Cergy, France). Bacterial cells were counted in a Salumbini chamber, and then adjusted to the desired concentration.

TAIL-PCR

The flanking region of gene sat was accessed by using the TAIL-PCR method (Liu and Whittier, 1995). This method uses a short arbitrary degenerate oligonucleotide previously described. To amplify the 5′-flanking region of the sat gene, three consecutive sequence specific oligonucleotides were used (5′-TACCGCGCAACAGCCAGAGACAC-3′, 5′-GAAGATCAAGGATGATGAGTG-3′, 5′-CTGTCTCCGCC-3′ and 5′-CTGCACCAGGAAATATTGCGGATTTG-3′). To amplify the 3′-flanking region of the sat gene, three consecutive sequence specific oligonucleotides were used (5′-CGGGTCTTGTGGAGAAATCAGGC-3′, 5′-GCGTTCTGCGAGAAAC-3′ and 5′-CGGTTGCTTGTGGAGAAATCAGG-3′). PCR fragments of the sat-flanking region obtained were cloned into TOPO-XL vector (Invitrogen), and sequenced using oligonucleotides provided in the Topo-cloning kit.

Southern blot analysis

Two different probes were used. A probe corresponding to 744 bp of the sat gene of strain CFT073 was generated by PCR using a set of oligonucleotides (5′-CTGATGATTGCAGCTGAGGATGTTG-3′ and 5′-GCAGATCACCACATTGTTTGTTG-3′). A second probe corresponding to 669 bp of the conserved C-terminal autotransporter region of SPAEs was generated by PCR using a set of oligonucleotides (5′-CAAAGCGTATGGTGACAGCCGACAGC-3′ and 5′-CATTCTCCGCGGACAGC-3′). Bacteria were cultured in LB. Genomic DNA was extracted using the
Nucleospin tissue’s kit (Macherey-Nagel, France) following the manufacturer’s instructions. Ten micrograms of genomic DNA was digested with EcoRV then separated on a 0.9% gel agarose. DNA was transferred onto a nylon nucleic acid transfer membrane (Hybond N+, Amersham Pharmacia Biotech, Buckinghamshire, UK), fixed by baking the membrane for 2 h at 80°C, and hybridized with the specific labelled sat probe. Revelation was carried out using the ECL direct nucleic acid labelling and detection system (Amersham Pharmacia Biotech) according to the manufacturer’s instructions.

Construction of vectors expressing gene sat

The satP1122 and satE777 genes were cloned into the pACYC184 vector (Rose, 1988) between EagI and BamH1 restriction enzyme sites using the set of oligonucleotides (5’-GACG GGGATCCCTCTCATGAtaATGAGAACATTGTC-3’ and 5’-CT AGAAccggccgctaataagagacagaccgat-3’). PCR was performed using pfu ultra DNA polymerase (Stratagen, Netherlands).

Production and identification of Sat protein

Cultures (1000 ml) of bacterial strains grown in LB (OD600 of 1) were used to purify Sat. Bacteria were removed by centrifuging at 12 000 g for 10 min at 4°C. Supernatants were filtered through a 0.22 µ pore-sized filter. Samples were concentrated using cross-filtration (10 000 molecular weight cut-off) to a volume of approximately 1 ml, and proteins were analysed by SDS-PAGE.

The bands nearest to the 107 kDa size were excised, subjected to in-gel trypsin digestion, and the resulting peptides analysed by MALDI-TOF mass spectrometry. Mass spectra of tryptic peptides were acquired using a PE Biosystems Voyager-DE STR mass spectrometer (Applied Biosystem, Les Ulis, France) with a nitrogen laser at 337 nm. Samples were prepared with an α-cyano-4-hydroxycinnamic matrix (Roche, Meylan, France). The spectra were acquired over the range 600–4000 Da, under reflector conditions with an accelerating voltage of 20 kV, and an extraction delay time of 125 ns.

Proteins were identified from the peptide mass spectra using the MASCOT peptide mass fingerprint software (version June 2005). Those with an estimated E-value of < 0.0001 were taken to be significant matches.

Site-directed mutagenesis

Site-directed mutagenesis was performed using the Quick change site-directed mutagenesis kit (Stratagen, Netherlands) to replace the serine at residue 260 of the serine protease motif by glycine using 0.02% trypsin in Ca2+Mg2+-free PBS containing 3 mM EDTA. Experiments and cell maintenance were carried out at 37°C in an atmosphere of 10% CO2–90% air. The culture medium was changed daily. For assays, fully differentiated cells were used at post confluence after 15 days in culture (Peiffer et al., 2000a,b).

Bacterial cell infection

The method used to infect the cells has been described elsewhere (Peiffer et al., 2000a,b). Briefly, the cell monolayers were washed twice with PBS. Infecting E. coli bacteria were suspended in culture medium, and a total of 10^8 cfu well^1 of this suspension was added to each well of the tissue culture plate. In the presence of wild-type strains, the infection assay was conducted in the presence of 1% mannose to prevent type-1 fimbriae-mediated binding. The plates were incubated at 37°C in 10% CO2–90% air for 3 h. The monolayers were then washed three times with sterile PBS. Each assay was conducted in triplicate with three successive passages of cultured cells.

Measurement of TER

Monolayers of Caco-2/TC7 cells were grown on filters (0.4 µm polyester, tissue culture-treated, Transwell Costar). After apical infection, the integrity of the confluent polarized monolayers was checked by measuring the TER with a volt-ohmmeter (Millicell-ERS; Millipore, Saint Quentin, France). The background of a free control filter was subtracted. Results are expressed as percent of control cells.

Permeability measurements

The permeability of Caco-2/TC7 cells monolayers was determined by a widely used and validated technique that measures the paracellular passage of water-soluble radioactive or fluorescent compounds of various sizes (14C-mannitol 182 kDa, and FSA 478 kDa), from apical to basolateral compartments of the chamber culture (Costar culture plate inserts; 0.4 µm porosity; 4 × 10^6 cells per filter) (Peiffer et al., 2000a).

To determine flux in the apical-to-basolateral direction, 0.25 µCi ml^-1 of 14C-mannitol and 200 µg ml^-1 of FSA was loaded to the apical side of the monolayer, and the cells were incubated for 1 h at 37°C. After the incubation period, the tracer concentrations in the apical and basolateral compartments were assayed. The concentrations of 14C-mannitol was determined by measurement in a scintillation counter. The fluorescence due to

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FSA was determined at an excitation wavelength of 485 nm, and an emission wavelength of 535 nm. Values were corrected for the background of the medium, as appropriate and are expressed as the percentage of tracers found in the basolateral compartment relative to the total tracer in both apical and basolateral compartments.

**Immunofluorescence**

For direct or indirect immunofluorescence labelling, cultured cells were prepared on glass coverslips, which were then placed in 24 well TPP tissue culture plates (ATGC, Marne la Vallée, France).

Decay accelerating factor mobilization around adhering Afa/Dr bacteria was detected by using a DAF monoclonal antibody 8D11 (dilution of 1:100) as described previously (Guignot et al., 2000b).

For the detection of TJs-associated proteins, cells were fixed in 3% paraformaldehyde in PBS pH 7.4 for 15 min at room temperature then treated with 50 mM NH4Cl for 10 min. Monolayers were permeabilized using 0.1% Triton X-100 in PBS for 4 min at room temperature, then washed twice with PBS and once with PBS containing 0.1% gelatin and 10% FCS. Primary antibodies were diluted in 0.1% gelatin in PBS (anti-ZO-1, anti-ZO-3, anti-claudin-1 and anti-occludin (at a dilution of 1:100, 1:100, 1:40 and 1:100 respectively). The coverslips were incubated with primary antibodies for at least 2 h, washed three times in PBS then incubated with the appropriate TRSC-conjugated secondary antibodies (at a dilution of 1:200) for at least 30 min. When F-actin was to be visualized, cells were permeabilized by incubating with 0.2% Triton X-100 in PBS for 4 min at room temperature before incubating with fluorescein-phalloidin for 45 min at room temperature (Peiffer et al., 2000b). The coverslips were then washed a further three times with PBS.

Specimens were mounted using Dako fluorescent mounting medium (DAKO), and examined using a CLSM (model LSM 510 Zeiss, equipped with an air cooled argon ion laser 488 nm, and a helium neon laser 543 nm) configured with an Axiovert 100 M microscope using a Plan Apochromat 63X/1.40 oil objective. Optical sectioning (horizontal optical sections) was used to collect 50 En face images. The reconstruction of successive En face micrographs obtained by CLSM analysis was performed using the accompanying Zeiss software LSM510 2.5 on Windows NT4. Photographic images were resized, organized and labelled using Adobe Photoshop software (San Jose, CA).

**Western blot analysis of TJs-associated proteins**

Following infection, the cells were washed once with cold PBS then treated for 15 min at 4°C with extraction buffer (25 mM HEPES, 0.5% triton, 150 mM NaCl, 2 mM EDTA) containing proteases and phosphatases inhibitors. Protein fractions were dissolved in the appropriate volume of Laemlli buffer and held at 100°C for 5 min. Proteins were immediately separated on 12% SDS-polyacrylamide gels for occludin and claudin-1, and 7% SDS-polyacrylamide gels for ZO-1 and ZO-3. For Western blot analysis, gels were transferred to polyvinylidene difluoride membrane (Perkin Elmer, Les Ulis, France)) and examined using the ECL+ detection system under conditions recommended by the manufacturer (Amersham). Incubation of membranes with primary anti-ZO-1, anti-ZO-3, anti-occludin or anti-claudin-1 anti-bodies was followed by incubation with horseradish peroxidase-conjugated with anti-rabbit or anti-mouse as secondary antibody.

Western blot signals were quantified using Scion image software (Scion Corporation, Frederick, MD) and densitometry data from at least four independent experiments were averaged for statistical analysis.

**Statistical analysis**

All experiments were repeated at least three times. The statistical significance was determined by the χ² test, and P < 0.01 was considered to be significant. For the analysis of the presence of the sat gene in the collections of Afa/Dr DAEC, a global F-test was used and P < 0.01 was considered to be significant.

**Acknowledgements**

We are particularly grateful to I. Scalaletsy (Department of Microbiology, Immunology, and Parasitology, Universidade Federal de Sao Paulo, Escola Paulista de Medicina, Sao Paulo, Brazil), A. Darfeuille-Michaud (Faculté de Médecine et de Pharmacie, Clermont-Ferrand, France), and C. Le Bouguenec (Institut Pasteur, Paris, France) for generous gifts of clinical Afa/Dr E. coli isolates. We thank R. Amsellem for her expert assistance with cell cultures. We thank M. Vasseur for statistical analysis. We are also indebted to C. Boursier for her kind help with MALDI-TOF mass spectrometry analysis [Plateforme Protéome (IFR141), Faculté de Pharmacie Paris XI]. We would like to thank B.J. Nowicki (Galveston, TX, USA), C. Le Bouguenec (Paris, France), and E. Oswald (Toulouse, France) for the gifts of prototypes of Afa/Dr DAEC and CFT073 strains. Our thanks also to D. Lublin (St Louis, MI, USA) for his gift of 8D11 antibody.

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