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Veterinary Microbiology 83 (2001) 275–286

veterinary microbiology

www.elsevier.com/locate/vetmic

Organisation and in vitro expression of *esp* genes of the LEE (locus of enterocyte effacement) of bovine enteropathogenic and enterohemorrhagic *Escherichia coli*

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Received 6 February 2001; received in revised form 14 June 2001; accepted 14 June 2001

Abstract

Enteropathogenic (EPEC) and enterohemorrhagic (EHEC) *Escherichia coli* infections are characterised by the formation of attaching and effacing (AE) lesions on intestinal epithelial cells. Secretion of extracellular proteins (EspA, EspB, and EspD) via a type III secretion apparatus is necessary for the formation of the AE lesions by human EPEC. In this study, we show that bovine EPEC and EHEC are also able to secrete polypeptides homologous to the already described Esp proteins, most probably via a type III secretion system. Bovine EPEC and EHEC strains present two different secretion profiles of Esp proteins which correlate to the pathotypes of the *esp* genes as determined by PCR. We also demonstrate that genes encoding secreted proteins, present in the LEE of two bovine strains, are organised in the same way as in the human EPEC strain E2348/69.

Keywords: Cattle *Escherichia coli*; Locus of enterocyte effacement; Protein secretion

1. Introduction

Enteropathogenic (EPEC) and enterohemorrhagic (EHEC) *Escherichia coli* cause a characteristic intestinal lesion known as attaching and effacing (AE) lesion (Moon et al., 1983), and are called ''attaching and effacing *E. coli*'' (AEEC). In addition to AE lesion, EHEC produce verotoxins (VT) or Shiga toxins (Stx) (reviewed in Nataro and Kaper, 1998; Mainil, 1999). The name EHEC is often restricted to human *E. coli* causing enterocolitis

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0378-1135/01/$ – see front matter
PII: S0378-1135(01)00418-7

and producing verotoxins; some authors include only such *E. coli* belonging to serogroup O157 (Nataro and Kaper, 1998). For the sake of clarity, the name EHEC will also be used in this manuscript for bovine strains producing AE lesions and Stx toxins. The name EPEC will be used for bovine strains producing AE lesions but no Stx toxins.

The AE lesion is characterised by the intimate attachment of the bacteria to the enterocyte membrane and by the effacement of the microvilli of the enterocyte. This lesion is associated with the accumulation of polymerised actin filaments underneath the adhering bacteria, leading to the formation of a pedestal-like structure (Finlay et al., 1992; Knutton, 1994). AEEC strains have been implicated in diarrhoea and dysentery in human and animals including 2–8-week-old calves (Chanter et al., 1984; Hall et al., 1985; Donnenberg and Kaper, 1992; Mainil et al., 1993; China et al., 1998).

In the human EPEC strain E2348/69 and EHEC strain EDL933, most of the genes required for the formation of the AE lesion are clustered on the bacterial chromosome forming a type III pathogenicity island called the locus of enterocyte effacement (LEE) (McDaniel et al., 1995; Perna et al., 1998). The LEE of strain E2348/69 is sufficient to confer AE activity in vitro, on cultured cells, when introduced into a non-pathogenic *E. coli* strain (McDaniel and Kaper, 1997). The LEE can be divided into three functional regions (Kaper et al., 1998). The left part contains genes (*esc* and *sep*) that encode for a type III secretion system responsible for the secretion of proteins EspA (24–25 kDa), EspB (37 kDa), EspD (39–40 kDa) (Jarvis et al., 1995; Jarvis and Kaper, 1996) and Tir (Kenny et al., 1997). The middle part of the LEE contains two genes (*eae*, *tir*) the products of which promote intimate attachment of bacteria to host cells and reorganisation of cytoskeletal actin underneath the adherent bacteria. The *eae* gene encodes a 94 kDa outer membrane protein, intimin, required for intimate adherence (Jerse et al., 1990). The *tir* gene encodes the intimin receptor (Tir), which is translocated into the eucaryotic cytoplasm where it becomes phosphorylated and then incorporated into the host cell membrane (Kenny et al., 1997). The right part of the LEE contains three genes: *espA*, *espD* and *espB*, encoding proteins that are essential for EPEC-mediated signal transduction events within the host cell (Foubister et al., 1994; Kenny and Finaly, 1995; Kenny et al., 1996; Lai et al., 1997). Several studies (Adu-Bobie et al., 1998; China et al., 1999; Oswald et al., 2000) have identified variants within the *eae*, *tir*, *espA*, and *espB* genes of EPEC and EHEC: five variants of the *eae* gene (α, β, γ, δ and ε) and three for each of the other genes (α, β, and γ).

The secreted proteins (EspA, EspB, EspD, and Tir) probably interact with specific chaperone proteins in the bacterial cytoplasm. Such chaperones have indeed been identified for EspD (CesD) and Tir (CesT) (Wainwright and Kaper, 1998; Elliott et al., 1999). Components of the type III secretion system and EspA form an apparatus for translocation of EspB, Tir and probably EspD into the host cells (Kenny et al., 1997; Knutton et al., 1998). The translocated EspB and EspD are integrated into the cytoplasmic membrane of the target cells and may form a pore allowing other molecules to enter the target cells (Wolff et al., 1998).

One O26 EHEC strain (413/89-1) isolated from a calf was able to secrete proteins of 104, 55, 54, and 37 kDa when grown in Luria–Bertani (LB) broth, and proteins of 104, 37, 25, and 22 kDa when grown in tissue culture medium (Ebel et al., 1996). The N-terminal

sequences of the 25 and 37 kDa proteins were found to be highly homologous to the EspA and EspB proteins of the human EPEC strain E2348/69.

The aim of this study was (i) to confirm the secretion of Esp proteins by bovine EPEC and EHEC carrying LEE-related structure that produce AE lesions in vivo (Goffaux et al., 1997; Stordeur et al., 2000); and (ii) to further characterise their LEE-related structure by studying the presence of genes encoding for the secreted proteins and for chaperones and their relative positions, using PCR assays.

2. Material and methods

2.1. Bacterial strains

Eight Belgian (Mainil et al., 1993), two British (A52, S102-9) (Chanter et al., 1986; Wray et al., 1989) and two American (193, 1431) (Mainil et al., 1987) EPEC or EHEC isolates from young calves with diarrhoea and one EPEC isolate (Ab28) from an 18-month-old bovine at the slaughterhouse (China et al., 1998) were included in the study (Table 1). Positive controls were bovine strain S102-9 (O5), which had been already studied for protein secretion (Ebel et al., 1996), the human EPEC strain E2348/69 (O127:H6) (Donnenberg and Kaper, 1991), the human EHEC strain ATCC 43888 (O157:H7), and the rabbit EPEC strain E22 (O103) (Camguilhem and Milon, 1989). The *E. coli* K12-HB101 strain was used as negative control.

Table 1
Pathotype of the studied strains and the positive controls

Strains	Origin	Serotype	Group	Results of multiplex PCR[a]				
				eae[b]	*tir*[b]	*espA*[b]	*espB*[b]	*espD*
329S89	Calf	Rough:H11	EHEC	β	β	β	β	β
330S89	Calf	O5:H-	EHEC	β	β	β	β	β
331S89	Calf	O15:H11	EHEC	β	β	β	β	β
340S89	Calf	O118:H16	EHEC	β	β	β	β	β
359S89	Calf	Rough:H2	EHEC	γ	α	α	α	α
361S89	Calf	Rough:H16	EHEC	γ	α	α	α	α
193	Calf	O26	EHEC	β	β	β	β	β
1431	Calf	O111:K58	EHEC	γ	α	α	α	α
Ab28	Bovine	ND[c]	EPEC	γ	α	α	α	α
A52	Calf	O26:H11	EHEC	β	β	β	β	β
48369MF1	Calf	ND[c]	EPEC	β	β	β	β	β
46919MF3	Calf	ND[c]	EPEC	γ	α	α	α	α
S102-9	Calf	O5	EHEC	β	β	β	β	β
E2348/69	Human	O127:H6	EPEC	α	α	α	α	α
E22	Rabbit	O103	EPEC	β	β	β	β	β
43888	Human	O157:H7	EHEC	γ	γ	γ	γ	γ

[a] According to the classification described by Adu-Bobie et al. (1998).
[b] China et al., 1999.
[c] ND: not determined.

2.2. PCR reactions

The variants of *espD* gene were identified using B186 as constant primer and B188, B187, and B189 as variable primers, which were able to amplify *espD*α-, *espD*β-, and *espD*γ-variant, respectively. Primers K518 and K519 (Wainwright and Kaper, 1998), and cesT-up and cesT-lo were used to amplify the complete *cesD* and *cesT* gene, respectively. Primers B163 and B214 amplified the region between *espA* and *espD* genes, primers F9 and B149 amplified the region between *espD* and *espB*β-type genes, and region *espD* to *espB*α-type genes was amplified using primers F9 and B151 (Table 2).

PCR reactions were performed in a Gene Cycler (Bio-Rad, Hercules, USA), using Taq DNA polymerase (Roche, Germany) as described previously (China et al., 1999). PCR products were analysed by electrophoresis in 1–2% agarose gel (Life Sciences International, Zellik, Belgium).

2.3. SDS–PAGE, microsequencing, and immunoblotting

Protein analysis was performed following an adaptation of the method described by Kenny et al. (1997). Strains were inoculated into 5 ml of LB broth and grown overnight at 37°C with shaking. Bacteria were diluted 1:50 and grown without shaking for 6 h in 5 ml Dulbecco's modified Eagle medium (DMEM) (Life Technologies, Rockville, MD, USA) at 37°C in a 6-well plate. A volume of 1.5 ml of culture was transferred in a 2 ml Eppendorf. Bacteria were pellted by centrifugation (18,000 × *g*, 10 min), and proteins in the supernatant were precipitated by the addition of trichloroacetic acid (TCA) (10% (v/v); Acros Organics, Geel, Belgium) for 60 min on ice. Precipitated proteins were pellted by

Table 2
Primers used in this study

Genes	Primers and sequences	Optimal annealing temperature (°C)[a]	Size of the PCR product (bp)
*espD*α	B186, 5′CGAAGAACAACAAAAAGCC3′; B188, 5′ACAGCAAAAGCAGAAACCT3′	52.8	492
*espD*β	B186, 5′CGAAGAACAACAAAAAGCC3′; B187, 5′GCAGAGGTCGTAAATCCAT3′	53.4	414
*espD*γ	B186, 5′CGAAGAACAACAAAAAGCC3′; B189, 5′CTGCCGCTTTCTCAACGAC3′	53.8	350
cesD	K518, 5′TTGGTATAGCTGATGGTAGTTTT3′; K519, 5′ATTAAAGCAACCCCGATAAAA3′	50.7	530
cesT	cesT-up, 5′ATGTCATCAAGATCTGAACTTTTA3′; cesT-lo, 5′TTATCTTCCGGCGTAATAATGTTT3′	50.4	471
espA to *espD*	B163, 5′TGAGGCATCTAARGMGTC3′; B214, 5′NTTTCTCTTCGGCTTTY3′	51.6	890
espD to *espB*β	F9, 5′TTGGTGGTATTAGTGGTGA3′; B149, 5′CTTTCCGTTGCCTTAGT3′	52.2	1381
espD to *espB*α	F9, 5′TTGGTGGTATTAGTGGTGA3′; B151, 5′TCCCCAGGACAGATGAGAT3′	52.2	1271

[a] As calculated by the Oligo software.

centrifugation at 4°C (16,000 × *g*, 10 min) and resuspended in 8 M urea and Laemmli sample buffer (Bio-Rad). Samples were resolved on 12% polyacrylamide gels (Bio-Rad) and were visualised by Coomassie R-250 blue staining. LMW Electrophoresis Calibration Kit (Amersham Pharmacia Biotech, Uppsala, Sweden) was used as molecular weight markers.

For N-terminal sequencing, the proteins were separated by SDS–PAGE and blotted onto PVDF membrane (Millipore, St. Quentin Yvelines, France) with a semi-dry transfer cell (Bio-Rad). After visualisation of the protein bands with Coomassie blue, bands of interest were cut out and analysed with a Protein Sequencer (model 492, Applied Biosystems, Foster City, CA, USA).

For immunoblotting, separated proteins were transferred to nitro-cellulose membranes (Bio-Rad) using mini trans-blot electrophoretic transfer cell (Bio-Rad). Membrane was blocked for 1 h with phosphate-buffered saline (PBS) supplemented with 5% (w/v) skim powdered milk and probed overnight with rabbit polyclonal EspB antiserum at a dilution of 1:1000. Following three washes with PBS containing 0,1% Tween 20 (Sigma, Bornem, Belgium), the membrane was incubated for 1 h with a 1:3000 dilution of peroxidase-conjugated swine anti-rabbit immunoglobulins (Dako, Glostrup, Denmark) in PBS supplemented with 5% skim powdered milk. The reaction was visualised with 4-chloro-1-naphtol (Sigma).

3. Results

3.1. PCR typing of eae, tir, and espADB genes

The *eae*, *tir*, *espA*, and *espB* genes of the bovine EPEC and EHEC were previously typed, using four multiplex PCR (China et al., 1999). To complete the pathotype, a similar approach was used to type the *espD* gene. The available sequences of *espD* gene from E2348/69 (representing α-variant; accession number GenBank AF022236), from E22 (representing β-variant; accession number GenBank AF054421), and from EDL933 (representing γ-variant; accession number GenBank AF071034) were aligned. An upper primer was selected within a constant part and a lower primer was selected within a variable part of each subtype to develop a multiplex PCR. An amplicon of 492 bp was obtained for five bovine strains as for strain E2348/69, and an amplicon of 414 bp was obtained for 8 bovine strains as for strain E22. No amplicon of 350 bp was observed excepted for the positive control strain 43888 (Table 1).

3.2. Genetic organisation of esp genes within the LEE of two bovine EHEC strains, and presence of genes encoding for chaperone proteins

The genetic organisation of the *esp* genes from two bovine EHEC strains presenting two different pathotypes (340S89 and 361S89) was compared to human EPEC strain E2348/69 by PCR amplifying the following intergene regions: *espA* to *espD*, and *espD* to *espB*. Primers were located in constant region of the genes excepted for *espB*: primers specific for α-type (361S89) and for β-type (340S89) were used. Amplification profiles

obtained with the two bovine strains were identical to the profile obtained with strain E2348/69.

In addition to genes encoding for secreted proteins, we tested by PCR the presence of two genes, *cesD* and *cesT,* encoding for the chaperone proteins of EspD and Tir, respectively. The 13 bovine strains were positive for the two *ces* genes, as was the positive control E2348/69.

3.3. Secretion of proteins by bovine EPEC and EHEC in tissue culture medium

Previous (Goffaux et al., 1997; China et al., 1999) and the above results confirmed that bovine EPEC and EHEC used in this study possess genes encoding for secreted proteins but did not prove their ability to secrete Esp-like proteins. For this purpose, culture supernatants were analysed by SDS–PAGE with strain S102-9 as a positive control. Secreted proteins were observed in all culture supernatants except strain Ab28 (Fig. 1). For strains 329S89, 330S89, 340S89, 193, A52, 48369MF1 and S102-9, four proteins of 100, 39, 37, and 25 kDa were detected; for strain 331S89, proteins of the same molecular weight, but the 100 kDa were present; and for strains 359S89, 361S89, 1431 and 46919MF3, four proteins of 100, 40, 37, and 25.5 kDa were visualised.

3.4. Identification of proteins secreted by bovine EPEC and EHEC

Detection of the EspB protein in culture supernatants of all bovine EPEC and EHEC strains was investigated by immunoblotting with a rabbit polyclonal EspB antiserum raised from strain E2348/69 (Dr. Gad Frankel's gift). One band reacting with EspB antibody was detected in all bovine strains as in the positive control E2348/69, except for Ab28 strain since this strain did not secrete any protein in the culture supernatant (Fig. 2).

To confirm the identity of the proteins observed on SDS–PAGE to Esp-like proteins, the N-terminal sequences of the secreted proteins from two strains presenting two different secretion profiles were determined. Sequences were obtained for three proteins from strain 340S89 (39, 37, and 25 kDa) and for three proteins from strain 361S89 (40, 37, and 25.5 kDa). N-terminal sequences were compared with the known sequences in the

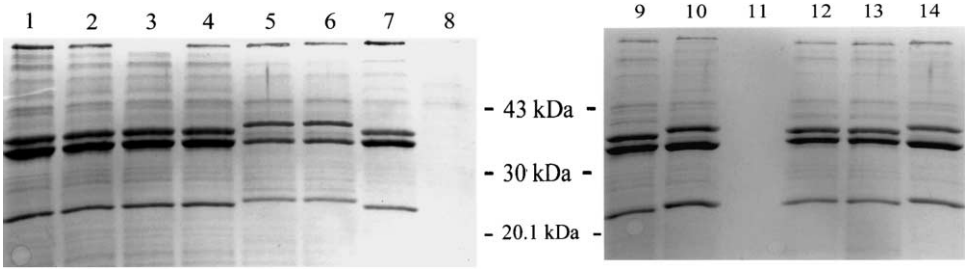

Fig. 1. Coomassie blue-stained SDS–PAGE gels of culture supernatants from 13 bovine EPEC or EHEC strains and *E. coli* K12-HB101. Lane 1: 329S89, lane 2: 330S89, lane 3: 331S89, lane 4: 340S89, lane 5: 359S89, lane 6: 361S89, lane 7: S102-9, lane 8: HB101, lane 9: 193, lane 10: 1431, lane 11: Ab28, lane 12: A52, lane 13: 48369MF1, lane 14: 46919MF3.

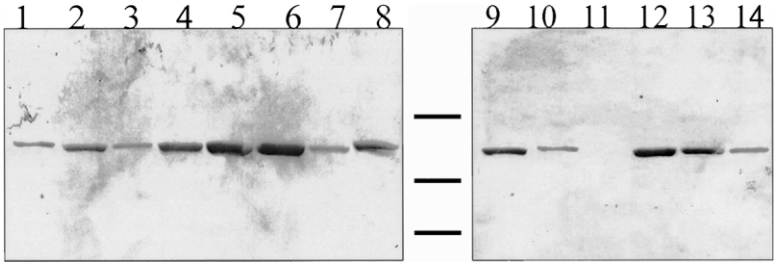

Fig. 2. Western immunoblot showing detection of EspB in the culture supernatant of bovine EPEC and EHEC strains using antibody to EspB. Lane 1: 329S89, lane 2: 330S89, lane 3: 331S89, lane 4: 340S89, lane 5: 359S89, lane 6: 361S89, lane 7: S102-9, lane 8: E2348/69, lane 9: 193, lane 10: 1431, lane 11: Ab28, lane 12: A52, lane 13: 48369MF1, lane 14: 46919MF3.

databases, using BLASTP (Altschul et al., 1997) (Table 3). The 25 kDa protein of 340S89 showed an amino terminal sequence similar to nine EspA proteins from different EPEC and EHEC strains, with 90% identity to the β-variants (EspA of strains RDEC-1 and E22). The 25.5 kDa protein of strain 361S89 was related by its N-terminal sequence to the EspA protein of an EPEC strain O55:H7 (90% identity). The 37 kDa protein of 340S89 had an

Table 3
Sequence identities of secreted proteins by two bovine strains (340S89 and 361S89) to known proteins in the databases[a]

Sequence	Protein/PCR variant	Accession number GenBank
MDTSTA**TSV**V	25 kDa (340S89) (β)	
MDTSTA**TSV**A	EspA RDEC-1 (β); EspA E22 (β); EspA 413/89-1; EspA O111:H2/(19); EspA O128:H2/(20); EspA 1390; EspA 4221	AAB71083; AAC82358; CAA74172; CAA12348; CAA12351; AAC99337; AAF26454
MDTSNA**TSV**V	EspA EDL933 (γ)	AAC31501
MDTSTG**TSV**A	EspA O119:H2/(79)	CAA12345
MNTIDYTNQV	37 kDa (340S89) (β)	
MNTIDYTNQV	EspB RDEC-1 (β); EspB E22 (β); EspB 413/89-1; EspB 4221; EspB 1390; EspB 84/110-1; EspB 83/39; EspB E65/56	AAB69980; AAC82360; CAA67984; AAD12778; AAC99339; AAD34582; AAD34583; AAD34584
MLNVNSDIIQ	39 kDa (340S89) (β)	
MLNVNSDIQS	EspD E22 (β); EspD 413/89-1; EspD 1390; EspD 4221; EspD DA-EPEC-B6	AAC82359; CAA74173; AAC99338; AAF26455; CAA76910
MDTLNTASVV	25.5 kDa (361S89) (α)	
MDTLNTASVA	EspA O55:H7	CAA12350
MNTIDNNNAA	37 kDa (361S89) (α)	
MNTIDNNNAA	EspB E2348/69 (α)	AAC38396
MLNVNSDIEQ	40 kDa (361S89) (α)	
MLNVNSDIQS	EspD E22 (β); EspD 413/89-1; EspD 1390; EspD 4221; EspD DA-EPEC-B6	AAC82359; CAA74173; AAC99338; AAF26455; CAA76910
MLNVNNDIQS	EspD E2348/69 (α); EspD DA-EPEC-3431	AAC38395; CAA76909

[a] Identical residues are in bold type, and homologous residues are underlined.

N-terminal sequence identical to eight EspB proteins from different EPEC and EHEC strains, with 100% identity to the β-variants (EspB of strains RDEC-1 and E22). The same molecular weight protein of strain 361S89 showed an N-terminal sequence 100% identical to the EspB protein of strain E2348/69 (α-variant). The 39 kDa protein of strain 340S89 and the 40 kDa protein of strain 361S89 differed only by the amino acid at position nine. The N-terminal sequence of the 39 kDa protein was similar to EspD proteins from different strains, with 80% identity to the sequence of the EspD protein of strain E22 (β-variant). The 40 kDa protein showed a sequence similar to seven known EspD proteins, with only 70% identity and 90% homology to the EspD protein of strain E2348/69 (α-variant).

4. Discussion

Human EPEC and EHEC are able to secrete at least four proteins in tissue culture medium: EspA (24–25 kDa), EspB (37 kDa), EspD (39–40 kDa), and EspC (110 kDa) (Jarvis et al., 1995; Kenny and Finaly, 1995; Jarvis and Kaper, 1996). In this study of bovine EPEC and EHEC carrying the LEE-related structure, we demonstrated the secretion of three Esp-like proteins and the existence of two secretion profiles. Three of the proteins detected for each strain with either profile had molecular weights closely similar, or identical, to the molecular weight of the EspA, EspB and EspD proteins secreted by human EPEC and EHEC, and two of them to the molecular weight of the EspA and EspB proteins secreted by bovine strains (Ebel et al., 1996). N-terminal sequencing of 10 amino acids of the proteins secreted by two bovine strains confirmed their relationship to EspA, EspB and EspD with 70–100% sequence identity. It was even possible to identify one variant (β) for the EspA protein of strain 340S89 and two variants (α and β) for the EspB proteins (90 and 100% sequence identities) (Table 3). The EspB protein, one of the most important factors involved in signal transduction events, was identified also by immunoblotting with a specific polyclonal antiserum in 12 bovine EPEC and EHEC strains, independently of the EspB variant.

The other important observation was the correlation of the two Esp protein profiles to the PCR profiles of the *esp* genes. The four strains with the *espα* genes gave one secretion profile and the eight strains with *espβ* genes gave the other secretion profile. In either secretion profile, one EspA and the EspB variants indeed correspond to the *esp* gene variants as identified by PCR. On the other hand, the EspA protein of strain 361S89 and the EspD proteins could not be clearly correlated with the corresponding *esp* gene variants. Comparison of larger sequences should be performed. Nevertheless, *espA* gene sequence of EPEC strain O55:H7 could be compatible with α-variant using our primers (data not shown).

Many type III secreted proteins require chaperones, whose role is to stabilise proteins and prevent inappropriate protein–protein interactions. Such a system is found in many pathogenic bacteria including enterobacteria (*E. coli, Salmonella*, *Shigella*, *Yersinia*) and in plant pathogens as well (reviewed in Hueck, 1998). A type III secretion system most probably also secretes the Esp-like proteins detected in this study, since previous hybridisation results with the LEE probes (Goffaux et al., 1997) and PCR assays (data not shown) showed that all the bovine strains possess sequences homologous to genes

encoding for components of the type III secretion apparatus. Moreover, genes encoding for chaperone proteins CesD and CesT are also present. Thus, the type III secretion system seems to be well conserved among human and bovine EPEC and EHEC. Its actual role in the secretion of the Esp protein of bovine EPEC and EHEC would be proved by creation of knock-out mutants in one of its genes, as already observed for human EPEC and EHEC (Jarvis et al., 1995; Jarvis and Kaper, 1996).

Recently, Mellies et al. (1999) showed that the components of the type III secretion system are transcribed from three polycistronic operons designated LEE1, LEE2 and LEE3. The secreted Esp proteins are part of a fourth polycistronic operon designated LEE4. In EHEC EDL933 strain, the *espADB* genes are also organised in a single operon (Beltrametti et al., 1999). Using our primers we showed that the *espADB* genes of two bovine EHEC are most probably organised as their homologues on the LEE of strain E2348/69. Therefore, *espADB* genes may also form a polycistronic operon like LEE4.

Eleven of the bovine EPEC and EHEC strains also secrete an additional 100 kDa polypeptide. In human EPEC, the 110 kDa polypeptide (EspC) has homology with the immunoglobulin A protease family. Mutation of the gene encoding this protein has no effect on AE lesion formation (Stein et al., 1996). Furthermore, the secretion of EspC in EPEC is not dependent on the *sep* transport genes (Jarvis and Kaper, 1996). Since, the 104 kDa polypeptide secreted by the bovine EHEC 413/89-1 is homologous to the N-terminal sequence of 110 kDa of EPEC E2348/69 (Ebel et al., 1996), the 100 kDa protein observed in our study is possibly also homologous to EspC. Unlike Ebel et al. (1996), we were unable to observe proteins of 55, 54, and 22 kDa, but the experimental conditions, more especially bacterial growth media, are different. There is no straightforward explanation for the fact that EPEC strain Ab28 did not secrete any protein in tissue culture medium although it was able to produce AE lesion in vivo (Goffaux et al., 1997). Explanations may include the requirement for a contact between the bacterium and epithelial cell for secretion or the need for specific conditions for secretion by this strain, or the occurrence of some mutations abolishing the secretion process in this strain since it was tested in vivo.

Development of multiplex PCR allowed us to type five of the most important genes implicated in the formation of the AE lesion (China et al., 1999; this study). Associating the results of *eae*, *tir, espA, espB* and *espD* gene typing in the 13 strains studied, only two pathotypes are observed: *eae*β–*tir*β–*espA*β–*espB*β–*espD*β and *eae*γ–*tir*α–*espA*α–*espB*α–*espD*α. Although homologous association between *eae* and *tir* genes (*eae*β associated with *tir*β) can be observed, heterologous association is also present (*eae*γ associated with *tir*α). Nevertheless, associations between *espA*, *espB,*and *espD* genes are only homologous. These results agree with the recent description of the implication of EspA in the translocation of EspB into the host cell (Knutton et al., 1998) and the implication of EspD in the formation of EspA containing filaments (Kresse et al., 1999). Interestingly, in one pathotype, *esp* genes are of the same type as *eae* and *tir* genes, whereas, in the other pathotype, *esp* genes are of the same type as the *tir* gene, but not the *eae* gene. This could be explained by the fact that the *esp* and *tir* genes encode for secreted and translocated proteins, but the *eae* gene, which encodes for a bacterial outer membrane protein, does not. There was no correlation between pathotype and belonging to the EPEC or EHEC group.

5. Conclusion

In conclusion, the results of this study confirm the presence in bovine EPEC and EHEC of all elements composing the different regions of the LEE described in human strains. Not only is this LEE structurally present, but it is also functional in vitro, secreting Esp-like proteins like the human strains, and in vivo, leading to the production of AE lesions (Goffaux et al., 1997; Stordeur et al., 2000). In addition, two Esp protein profiles are observed which correlate with the *esp* gene PCR profiles. Furthermore the LEE of bovine EPEC and EHEC appear to be organised like the LEE of human EPEC and EHEC.

Acknowledgements

Frédéric Goffaux is a postgraduate of the FRIA (Fonds de la Recherche appliquée à l'Industrie et à l'Agriculture). This work was supported in part by Grant 5740A from the ''Ministère des Classes Moyennes et de l'Agriculture''. The authors thank especially Dr. Gad Frankel (Department of Biochemistry, Imperial College of Science, Technology and Medicine, London) for the gift of the EspB antiserum and Nicole Gérardin (Laboratoire de Biochimie, Université de Liège) for the N-terminal sequencing.

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