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A new pathogenicity island carrying an allelic variant of the Subtilase cytotoxin is common among Shiga toxin producing *Escherichia coli* of human and ovine origin

V. Michelacci[1,2], R. Tozzoli[1], A. Caprioli[1], R. Martínez[3], F. Scheutz[4], L. Grande[1], S. Sánchez[5] and S. Morabito[1]

1) *European Reference Laboratory for Escherichia coli, Istituto Superiore di Sanità, Dipartimento di Sanità Pubblica Veterinaria e Sicurezza Alimentare, Rome, Italy,* 2) *Department of Biology, University 'Roma Tre', Roma, Italy,* 3) *Patologia Infecciosa y Epidemiologia, Departamento de Sanidad Animal, Facultad de Veterinaria, Universidad de Extremadura, Cáceres, Spain,* 4) *WHO Collaborating Centre for Reference and Research on Escherichia and Klebsiella, Department of Microbiology and Infection Control, Statens Serum Institut, Copenhagen, Denmark and* 5) *Laboratorio de Enterobacterias, Servicio de Bacteriologia, Centro Nacional de Microbiologia, Instituto de Salud Carlos III, Madrid, Spain*

Abstract

Subtilase (SubAB) is a cytotoxin elaborated by some Shiga Toxin (Stx)-producing *Escherichia coli* (STEC) strains usually lacking the locus of enterocyte effacement (LEE). Two variants of SubAB coding genes have been described: $subAB_1$, located on the plasmid of the STEC O113 98NK2 strain, and $subAB_2$, located on a pathogenicity island (PAI) together with the *tia* gene, encoding an invasion determinant described in enterotoxigenic *E. coli*. In the present study, we determined the entire nucleotide sequence of the PAI containing the $subAB_2$ operon, termed Subtilase-Encoding PAI (SE-PAI), and identified its integration site in the *pheV* tRNA locus. In addition, a PCR strategy for discriminating the two *subAB* allelic variants was developed and used to investigate their presence in *E. coli* strains belonging to different pathotypes and in a large collection of LEE-negative STEC of human and ovine origin. The results confirmed that *subAB* genes are carried predominantly by STEC and showed their presence in 72% and 86% of the LEE-negative strains from human cases of diarrhoea and from healthy sheep respectively. Most of the *subAB*-positive strains (98%) identified possessed the $subAB_2$ allelic variant and were also positive for *tia*, suggesting the presence of SE-PAI. Altogether, our observations indicate that $subAB_2$ is the prevalent SubAB-coding operon in LEE-negative STEC circulating in European countries, and that sheep may represent an important reservoir for human infections with these strains. Further studies are needed to assess the role of *tia* and/or other genes carried by SE-PAI in the colonization of the host intestinal mucosa.

Keywords: STEC, pathogenicity island, subtilase cytotoxin, diarrhoea, small ruminants, PCR
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Corresponding author: S. Morabito, Dipartimento di Sanità Pubblica Veterinaria e Sicurezza Alimentare, Istituto Superiore di Sanità, Viale Regina Elena 299, 00161 Rome, Italy
E-mail: stefano.morabito@iss.it

Introduction

Subtilase (SubAB) is an AB_5 toxin produced by certain *Escherichia coli* strains associated with human disease [1]. SubAB is composed of a 35 kDa A subunit displaying a subtilase-like serine protease activity and five 13 kDa B subunits forming a pentamer, which mediates the binding to specific receptors on the host cell surface [1]. Following internalization in cultured cells, SubAB is delivered to the endoplasmic reticulum (ER) [2], where it has been demonstrated to cleave the chaperone BiP [3], causing the RNA-dependent protein kinase-like ER kinase activation and the transient inhibition of protein synthesis, resulting in the induction of the apoptotic signalling pathways [4–7].

SubAB has so far been identified almost exclusively in Shiga toxin (Stx)-producing *E. coli* (STEC), and in particular in strains that do not possess the locus for enterocyte effacement (LEE)

[8–18]. The LEE is a pathogenicity island (PAI) governing the attaching and effacing mechanism of intestinal adhesion [19], and represents a common feature of STEC strains associated with severe human disease. It has been hypothesized that the SubAB may contribute to the pathogenesis of STEC-associated human disease by playing a synergistic role with Stx [2]. As a matter of fact, SubAB has been shown to induce, in a mouse model, the typical haemolytic uraemic syndrome (HUS)-associated features caused by Stx, such as extensive microvascular damage, and thrombosis and necrosis in the brain, kidneys and liver [20].

The prototype SubAB-positive STEC strain 98NK2, belonging to serotype O113:H21 and isolated from an outbreak of HUS in South Australia [1], carries the subtilase-coding operon (*subAB*) on a large virulence plasmid designated as pO113, which also carries the *saa* gene, encoding an autoagglutinating adhesin possibly involved in the colonization of the host intestinal mucosa [21].

Recently, we reported the production of SubAB by two Stx-negative *E. coli* strains (ED 32 and ED 591), isolated from two unrelated cases of uncomplicated diarrhoea in Italy [22]. Genetic analyses showed that the nucleotidic sequences of the *subA* and *subB* genes were identical in the two strains and 90% similar to those of the corresponding genes present in the pO113 plasmid of strain 98NK2 [22]. Strains ED 32 and ED 591 were both LEE-negative and did not react in a *saa*-specific PCR assay. Moreover, differently from strain 98NK2, they harboured the *subAB* genes in the chromosome and next to another gene, *tia*, encoding an invasion factor previously described in enterotoxigenic *E. coli* (ETEC) [23]. An identical chromosomal region carrying *subAB* and *tia* was identified in the chromosome of other *subAB*-positive STEC strains [22], suggesting the existence of a putative pathogenicity island (PAI) vehiculating the *subAB* and *tia* virulence genes.

The presence of *subAB* genes among *E. coli* strains of human or animal origin has been investigated in several studies [8–18]. However, most of these studies did not involve the use of tools capable of distinguishing between the two allelic variants of the *subAB* gene. As an exception, a recent investigation conducted on STEC strains isolated from cattle, sheep and goats [14] reported a different distribution of the two *subAB* variants in the different animal species, with the $subAB_{98NK2}$, named by the authors $subAB_1$, associated with bovine strains, and $subAB_{ED32}$, termed $subAB_2$, more frequent among strains from small ruminants [14].

In the present work, we investigated the presence of the two allelic variants of the *SubAB* gene in human *E. coli* strains belonging to different pathotypes and in a large collection of LEE-negative STEC of human and ovine origin. Moreover, we determined the entire nucleotidic sequence of the putative PAI containing the $subAB_2$ operon in the prototype *E. coli* strain ED 32 and describe its gene content and insertion site.

Materials and Methods

Bacterial strains

The prototype *E. coli* strain ED 32, containing the $subAB_2$ allelic variant, is part of the culture collection of the Istituto Superiore di Sanità and has been previously described [22]. The panel of human strains investigated included 177 STEC strains belonging to 10 different serogroups and displaying different combinations of *stx*-coding genes, 26 enteropathogenic *E. coli* (EPEC), 32 enterotoxigenic *E. coli* (ETEC), 20 enteroaggregative *E. coli* (EAEC), 13 enteroinvasive *E. coli* (EIEC), all isolated from cases of diarrhoea, and one strain isolated from a urinary tract infection (uropathogenic *E. coli*, UPEC).

The diarrhoeagenic *E. coli* used in this study have been classified based on the presence of the virulence genes described to be associated to the different pathotypes in the literature. EPEC pathotype was identified by the presence of the intimin-coding *eae* gene, detected together with the plasmid-associated marker EAF for identifying typical EPEC. STEC were recognized by the presence of the *stx1* and *stx2* genes. The invasion plasmid antigen-coding gene *ipaH* and the enteroaggregative *E. coli* antiaggregation protein transporter gene *aat* (previously reported as CVD 432) were considered markers for EIEC and EAEC pathotypes respectively. Finally, ETEC strains were identified by the presence of the heat-stable and the heat-labile enterotoxin-coding genes (*est* and *elt*, respectively). All the virulence genes were amplified as previously described [22]. The PCR assay for the gene encoding the EAF determinant has been described in [24]. The only human UPEC was isolated from a patient suffering from urinary tract infection. Serotyping, including both O and H antigen identification, was performed by the WHO Collaborating Centre for Reference and Research on *Escherichia* and *Klebsiella* at the Statens Serum Institut (Copenhagen, Denmark).

All the STEC strains selected for this study lacked the *eae* gene, considered as a hallmark for the presence of the LEE locus. All the *E. coli* strains of human origin included in the study are part of the Statens Serum Institut (Copenhagen, Denmark) culture collection.

One hundred and twenty-three LEE-negative STEC strains isolated from sheep were included in the study. The strains had been isolated from healthy animals in Spain during a previous longitudinal study involving 12 different sampling visits (one sampling/month) at four different farms. All the animal

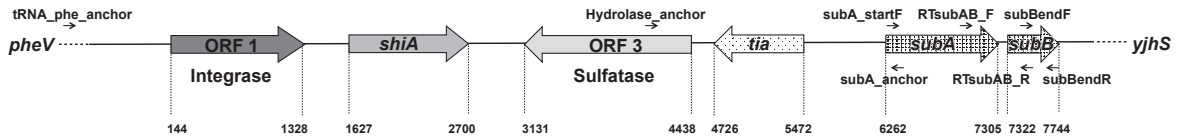

FIG. 1. Map of the Subtilase-Encoding Pathogenicity Island (SE-PAI), spanning the 8058 bp situated between the pheV tRNA locus and the *E. coli* gene yjhS. The ORFs and the annotated genes, together with their positions in the PAI sequence, are shown (GenBank Acc. No. JQ994271). The locations of the primers used to characterize SEPAI are also indicated.

TABLE 1. PCR primers used in this study for the characterization of the SubAB-encoding PAI, for the detection of *subAB*, *tia* and *saa*, and for identifying the two allelic variants of the *subAB* operon

Primer name	Gene target	Sequence	GenBank accession number (nucleotide position)	Reference
RTsubABF	*subA*	5′-GCAGATAAATACCCTTCACTTG-3′	AF399919 (13856-13835)	1
RTsubABR	*subB*	5′-ATCACCAGTCCACTCAGCC-3′	AF399919 (13625-13643)	1
tia lo	*tia*	5′-TCCATGCGAAGTTGTTATCA-3′	U20318 (577-558)	22
tia up	*tia*	5′-GAAATGAAAAAGATTATTGCGG-3′	U20318 (7-28)	22
SubAF	*subA*	5′-GTACGGACTAACAGGGAACTG-3′	AF399919 (14944-14964)	1
subA_anchor	*subA*	5′-CATGACGTGAGGCAATGAGT-3′	JQ994271 (6565-6545)	This study
tRNA_phe_anchor	*tRNAphe*	5′-ATTGAAAATCCCCGTGTCCT-3′	CU928164 (4802156-4802137)	This study
subA_startF	*subA*	5′-CCCTGTAACATATTGACCAGCA-3′	JQ994271 (6208-6230)	This study
subB_end_F	*subB*	5′-GGCCTTCGTTTTCCACATTA-3′	JQ994271 (7550-7570)	This study
subB_end_R	*subB*	5′-TTATGAGTTCTTTTTCCTGTCAGG-3′	AF399919 (13283-13306)	22
saa up	*saa*	5′-CGTGATGAACAGGCTATTGC-3′	AF399919 (7712-7731)	25
saa lo	*saa*	5′-ATGGACATGCCTGTGGCAAC-3′	AF399919 (7830-7811)	25

isolates are part of the culture collection held at the University of Extremadura (Caceres, Spain).

Construction of a genomic library from the ED 32 strain

A genomic library of strain ED 32 was constructed by using a Lambda ZAP II predigested *Eco*RI/CIAP-treated vector kit (Stratagene, La Jolla, CA, USA), following the supplier's instructions. In detail, 200 ng of total DNA were extracted from strain ED 32 (PureGene; Gentra Systems, Big Lake, MN, USA), partially digested with the *Eco*RI restriction enzyme, and ligated with 1 μg of the vector by incubation with 2 units of DNA ligase for 18 h at 4°C. Two μL of the Gigapack III gold packaging extract (Stratagene) were added to the ligase reaction mixture and incubated at room temperature for 2 h. Following addition of 500 μL of SM buffer (5.8 g/L NaCl, 2.0 g/L $MgSO_4$ $7H_2O$, 50 mM Tris-HCl (pH 7.5), 0.01% (wt/vol) gelatin) and 20 μL of chloroform, the supernatant containing the phages was titrated in *E. coli* XL1-Blue MRF' host bacteria. Amplification of the library was performed to obtain a final titre of 1.5×10^9 PFU/mL.

Identification of the subAB-flanking regions in the ED 32 strain

The sequence of the chromosomal regions flanking the *subAB* and *tia* genes in strain ED 32 was determined by using two different approaches. As the *tia* gene associated with $subAB_2$ shared 90% of sequence identity with the homologous gene in the ETEC strain IAI39, the region downstream this gene was obtained by PCR using the primer *tRNA_phe_anchor* designed on the available genomic sequence of this latter strain (GenBank Acc. No. CU928164), in combination with the *subA_anchor* primer, deployed on the sequence of the ED 32 strain *subA* gene (Fig. 1 and Table 1). The amplification reaction was conducted on a total DNA preparation from strain ED 32 and gave an amplification product of about 6,600 bp, which was subjected to sequencing.

The DNA sequence of the region located downstream the *subAB* operon was determined by sequencing the insert of a clone identified by screening the ED 32 library with a DNA probe corresponding to the 3′ terminal part of the *subB* gene obtained by PCR amplification using the subBendF/subBendR primer pair (Table 1) [22 and this study].

Evaluation of the activity of the two variants of SubAB by Vero cell assay

To investigate the possible differences in the activity of the two SubAB variants, their ability of inducing CPE onto Vero cell monolayers was compared by Vero cell assay (VCA). The culture supernatant from the *E. coli* strains ED 186 and ED 32, producing the $SubAB_1$ and $SubAB_2$ respectively, was assayed by inoculating serial dilutions, up to 1:1024, onto Vero cell monolayers in microtitre plates. As the ED 186 strain also produced the Stx1, each dilution of the culture supernatant from this strain was neutralized by incubation with an antibody raised against Stx1, at a working titre of 1:200, before inoculation. The VCA was conducted as previously described

TABLE 2. Summary of ORFs in SE-PAI of the ED 32 strain identified by significant similarity (BLASTP search)

ORF#	Location in SE-PAI	Denomination in SE-PAI	Closest informative protein match	Homologues Acc. No.	Aminoacidic (AA) identities	Positive AA substitutions
1	144-1328	integrase	site-specific recombinase, phage integrase family [*Escherichia coli* 99.0741]	ZP_11999107	394/394 (100%)	394/394 (100%)
2	1627-2700	*shiA*	putative homolog to shiA (SHI-2 pathogenicity island of *Shigella flexneri*) [*Escherichia coli* IAI39]	CAR19509	320/353 (91%)	332/353 (94%)
3	3131-4438	sulphatase	sulphatase family protein [*Escherichia coli* STEC_DG131-3]	ZP_12260098	431/435 (99%)	432/435 (99%)
4	4726-5472	*tia*	tia invasion determinant [*Escherichia coli* 1.2264]	ZP_11990814	247/248 (99%)	248/248 (100%)
5	6262-7305	*subA*	subtilase family protein [*Escherichia coli* STEC_EH250]	EGW88118	347/347 (100%)	347/347 (100%)
6	7322-7744	*subB*	subtilase cytotoxin subunit B [*Escherichia coli*]	ACV40235	140/140 (100%)	140/140 (100%)

[22] and the CPE observed at 24, 48 and 72 h after inoculation. The *subAB* genes from the two strains were also cloned in the pGEM-T-Easy vector and used to transform the K-12 strain JM109. The *subAB* operons were cloned together with their native promoters to allow the expression of the subtilase. Serial dilutions of overnight culture supernatants of the K-12 strains containing the cloned *subAB* operons were inoculated onto Vero cell monolayers after sonication of the culture to facilitate the release of the toxin produced and analysed in parallel with the wild-type strains in the same conditions.

PCR screening for the presence of subAB, saa and tia genes

The presence of *subAB* was assessed using the primer pair RTsubABF/RTsubABR [1], which allows the detection of both the allelic variants [22]. The *subAB* operon from the positive strains was typed using the primer pairs SubAF/RTsubABR [this study and 1] and subA_startF/RTsubABR [this study and 1], able to specifically detect the prototype *subAB* ($subAB_1$) or the allelic variant present in the ED 32 strain ($subAB_2$) respectively. The presence of *saa* and *tia* genes was assessed as previously described [22]. All the primers used are listed in Table 1.

Nucleotide sequence accession number

The DNA sequence of the 8,058 bp DNA stretch spanning the complete sequence of the PAI vehiculating *subAB* in the *E. coli* strain ED 32 was submitted into GenBank with the Acc. No. JQ994271. Annotation was made through the Glimmer online tool (http://www.ncbi.nlm.nih.gov/genomes/MICROBES/glimmer_3.cgi) and the predicted open reading frames were analysed with the blastp algorithm using the tool available at NCBI (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi?PROGRAM=blastp&BLAST_PROGRAMS=blastp&PAGE_TYPE=BlastSearch&SHOW_DEFAULTS=on&LINK_LOC=blasthome) in order to investigate the closest informative matches (Table 2).

All the nucleotidic alignments were carried out with the blastn algorithm using the tool available at NCBI (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi?PROGRAM=blastn&BLAST_PROGRAMS=megaBlast&PAGE_TYPE=BlastSearch&SHOW_DEFAULTS=on&LINK_LOC=blasthome).

Results

Characterization of the genomic locus harboring the $subAB_2$ operon in strain ED 32

The nucleotidic sequence of the genomic island vehiculating the $subAB_2$ operon in strain ED 32, partially described in a previous study, was completed and the structure of the locus is schematically reported in Fig. 1. Sequence analysis showed that the locus, that we termed Subtilase-Encoding PAI (SE-PAI), spans the 8 kb region between the *E. coli* gene *yjhS*, located 314 bp downstream of the $subAB_2$ operon, and the *pheV* tRNA locus, situated 4,725 bp from the end of the *tia* gene (Fig. 1). The latter DNA region contains an unnamed gene encoding a sulphatase, the *shiA* gene, whose product is involved in the attenuation of the *Shigella flexneri*-induced inflammatory response, and the genetic determinant for an integrase, probably involved in the PAI mobilization machinery (Table 2). Downstream the $subAB_2$ operon, SE-PAI, ends before the *yjhS* gene, which is part of an operon, named *yjhATS* or *nanCMS*, encoding factors involved in the internalization and catabolism of sialic acids. Such an operon was described in both commensal and pathogenic bacteria and is also present in the *E. coli* K12 reference strain MG1655 (GenBank Acc. No. NC000913). Therefore, it was considered as the downstream boundary of SE-PAI.

The whole sequence of the SE-PAI has been searched for homology with the records of the nucleotidic sequences, including the whole genomes, stored in GenBank, with the aim of investigating the variability of the ORFs and intergenic sequences of the PAI. Three contigs deriving from whole genome sequencing projects of STEC (*E. coli* 1.2264, Acc. No. AEZO02000028; *E. coli* DG131-3, Acc. No AFDV01000051; *E. coli* 9.0111, Acc. No. AEZZ02000022) contained the complete DNA sequence of the SE-PAI. In all the cases the sequences with a coverage of more than 90% shared between 97% and 99% of sequence similarity with the one identified in the prototype strain ED 32, indicating a conserved structure for this PAI. Interestingly, the STEC strains fully sequenced were isolated from small ruminants (*E. coli* 1.2264 and *E. coli*

DG131-3) and from a human case of infection (*E. coli* 9.0111). The alignment was also conducted against the whole sequence of the pO113 plasmid from the STEC O113:H21 strain EH 41 (Acc. No. NC_007365) and involved the entire SE-PAI and the DNA region downstream of the $subAB_2$ genes, corresponding to the *E. coli yjhATS* operon. The analysis showed that no DNA regions from the PAI or the flanking region were present in the plasmid harbouring the prototype $subAB_1$ allele.

Comparative analysis of the activity of the two SubAB variants

The Vero cell assays, conducted to evaluate possible differences in the activity of the two SubAB variants, showed that the Subtilase cytotoxin produced by both the alleles was able to induce the CPE up to a dilution of 1:128. This observation is in line with previous descriptions for the product of $subAB_2$ allele. Comparable results were also obtained for the two SubAB variants when the supernatant of the K12 strains containing the *subAB* recombinant plasmids was used in the VCA. In this latter case, however, the effect on the cells was visible only up to a dilution of 1:32, possibly caused by a lower efficiency of expression in the transformants compared with the wild-type strains.

Presence of the subAB genes in human pathogenic *E. coli*

The presence of *subAB* genes was investigated in a panel of 107 *E. coli* strains belonging to six different pathotypes and isolated from cases of human disease (Table 3). The association of *subAB* with LEE-negative STEC has previously been assessed in several investigations [10, 22, 24], therefore only STEC strains lacking the *eae* gene were selected for this study. The screening was carried out by PCR using a set of oligonucleotides (RTsubABF/RTsubABR, Table 1) able to prime the amplification of both the *subAB* allelic variants and revealed the presence of *subAB* genes in nine out of the 15 STEC (60%) and one of the 32 ETEC (3%) strains tested. All the strains belonging to the other pathotype were negative. The *subAB*-positive STEC belonged to eight of the ten serogroups tested (Table 3), and all possessed the $subAB_2$ variant, with the exception of one strain, which did not react with the primer pairs specific for the two *subAB* variants. No association between the presence of specific *stx*-gene types and the presence of *subAB* could be established (data not shown).

Detection and characterization of the subAB locus in LEE-negative STEC from human cases of diarrhoea

On the basis of the results obtained in the preliminary screening, 162 additional LEE-negative STEC from human cases of diarrhoea and belonging to the ten serogroups previously tested were selected and assayed by PCR for the presence of the two allelic variants of *subAB*, *tia* and *saa* (Table 4). The *subAB* genes were detected in about 72% of the strains, and the $subAB_2$ variant was present in 98.3% of the positive isolates. The $subAB_2$ genes were always associated with *tia*, with the exception of five O91 strains. An O181 strain was positive for both *subAB* and *tia*, but did not react in the primers specific for the two *subAB* variants, suggesting that additional *subAB* alleles may exist. Finally, an O91 strain was positive for both the $subAB_1$ and $subAB_2$ variants, as well as for the *saa* and *tia* genes, suggesting the concomitant presence of the SE-PAI and a plasmid similar to that described in the prototype O113 strain 98NK2. All the other strains were negative for *saa*.

PCR amplification of *tia* yielded a 600 bp product in the majority of the positive isolates, as expected on the basis of the published sequence of the reference ETEC strain IAI39 (GenBank Acc. No. U20318). A 1.8 kb PCR fragment was observed for 21 strains (19 O128, one O91 and one O146). This amplicon size is consistent with the presence of a 1.2 kb IS2 element interrupting the *tia* sequence, as has been previously described in strain ED 591 (GenBank Acc. No. FJ664545). Two additional strains (one O91 and one O146)

TABLE 3. Results of the PCR analyses for the presence of *subAB* in human *E. coli* strains belonging to different pathotypes (tEPEC, typical EPEC; aEPEC, atypical EPEC)

E. coli pathotype (No. of strains)	Serogroup (No. of strains)	No. of subAB-positive strains (allelic variant)
STEC (15)	O76 (1)	1 ($subAB_2$)
	O78 (1)	1 ($subAB_2$)
	O91 (2)	1 ($subAB_2$)
	O113 (1)	1 ($subAB_2$)
	O117 (2)	0
	O128 (2)	2 ($subAB_2$)
	O146 (2)	1 ($subAB_2$)
	O174 (1)	0
	O181 (1)	1 (untypeable)
	Orough (2)	1 ($subAB_2$)
tEPEC (3)	O119 (2)	0
	O145 (1)	0
aEPEC (23)	O8 (1)	0
	O26 (2)	0
	O55 (2)	0
	O103 (3)	0
	O111 (1)	0
	O114 (1)	0
	O119 (1)	0
	O125 (1)	0
	O127 (1)	0
	O128 (3)	0
	O145 (3)	0
	O157 (4)	0
ETEC (32)	O nt[a] (1)	0
	O6 (3)	0
	O8 (3)	0
	O25 (4)	0
	O44 (1)	0
	O153 (4)	0
	O164 (1)	0
	Orough (15)	1 ($subAB_2$)
EAEC (20)	O25 (1)	0
	O92 (2)	0
	O+[b] (17)	0
EIEC (13)	O121 (1)	0
	O nt[a] (12)	0
UPEC (1)	O102 (1)	0

[a]nt: not tested.
[b]O+: the O antigen is present but couldn't be typed.

TABLE 4. Results of the PCR analyses for the presence of the *subAB* allelic variants, *tia* and *saa*, in LEE-negative STEC strains of human origin

Genotype				No. of strains positive for				
Serotype	*stx1*	*stx2*	No. of strains	*subAB*	*subAB*$_2$	*tia*	*subAB*$_1$	*saa*
O76:H19	+	–	10	10	10	10	0	0
O78:H-	+	–	3	3	3	3	0	0
O78:H-	+	+	1	1	1	1	0	0
O91:H-	+	–	11	1	1	0	0	0
O91:H-	–	+	1	1	1	1	0	0
O91:H-	+	+	30	30	29	25	1[a]	1[a]
O113:H4	+	+	6	5	5	5	0	0
O117:H7	+	–	15	0	–	–	–	–
O117:H-	+	–	4	0	–	–	–	–
O128:H2	–	+	27	26	26	26	0	0
O128:H2	+	+	4	4	4	4	0	0
O146:H21	+	+	26	24	24	24	0	0
O146:H28	–	+	5	2	2	2	0	0
O146:H28	+	+	2	2	2	2	0	0
O174:H21	–	+	4[b]	0	–	–	–	–
O181:H16	+	–	3	3	2	3	0	0
Orough:H-	+	–	6	1	1	1	0	0
Orough:H-	+	+	4	4	4	4	0	0
Total			162	117	115	111	1	1

[a]One single strain was positive for *subAB*$_1$, *subAB*$_2$, *saa* and *tia* simultaneously.
[b]The strains have not been tested for the *subAB* variants, *tia* and *saa*, being negative to the *subAB* screening assay (see text for details).

TABLE 5. Results of the PCR analyses for the presence of the *subAB* allelic variants, *tia* and *saa*, in LEE-negative STEC strains isolated from healthy sheep

		No. of strains positive for				
Serotype	No. of strains	*subAB*	*subAB*$_2$	*tia*	*subAB*$_1$	*saa*
O5	11	8	8	8	0	0
O6	7	5	4	5	0	0
O76	12	12	12	11	0	0
O87	10[a]	0	–	–	–	–
O91	26	26	26	23	0	0
O123	5	5	4	5	0	0
O128	6	6	6	6	0	0
O146	19	19	19	19	0	0
O166	14	14	14	0	0	0
O176	13	13	13	12	0	0
Total	123	108	106	89	0	0

[a]The strains have not been tested for the *subAB* variants, *tia* and *saa*, being negative in the *subAB* screening assay (see text for details).

yielded both the 600 bp and 1.8 kb amplicons, suggesting the presence of two copies of *tia*, one of them possibly interrupted by the IS2 element.

Detection and characterization of the subAB locus in LEE-negative STEC from healthy sheep

The high prevalence of the *subAB*$_2$ variant in LEE-negative STEC isolated from patients with diarrhoea prompted us to investigate the possible animal reservoir of such *E. coli* strains. As the presence of the *subAB*$_2$ allele has been previously reported in small ruminants, a panel of 123 LEE-negative STEC isolated from sheep and belonging to 10 different serogroups was examined for the presence of the two *subAB* variants, as well as for *tia* and *saa* genes. The presence of *subAB* genes was detected in 108 strains (87.8%), all but two possessing the *subAB*$_2$ variant (Table 5). The latter two strains belonged to serogroups O6 and O123 and did not react with the primer pairs specific for the two *subAB* variants, further supporting the existence of additional *subAB* alleles.

As observed for the human strains, most of the *subAB*$_2$-positive ovine isolates (81.5%) were also positive for *tia*. The 19 *tia*-negative strains belonged to serogroups O166 (14 strains), O91 (three strains), O76 (one strain) and O176 (one strain). None of the strains was positive in the *saa*-specific PCR assay.

Discussion

Since their first description, SubAB-coding genes have been mainly detected in LEE-negative STEC [8–18], with the exception of two *E. coli* strains that did not produce Stx [22]. In the latter strains, the subtilase was encoded by an allelic variant of the *subAB* genes, termed *subAB*$_2$, which shared 90% homology with the prototype genes, *subAB*$_1$, identified in the virulence plasmid of the LEE-negative O113 STEC strain 98NK2 [2]. The *subAB*$_2$ operon was located on the chromosome, in a putative PAI that contained also the gene *tia*, encoding an invasion determinant described in ETEC [23]. In the present study, we completed the sequence of this putative PAI, which we termed SE-PAI, and showed that it is composed of an 8 kb DNA region inserted downstream the *pheV*-tRNA locus in the chromosome of strain ED 32. Beside *subAB*$_2$ and *tia*, SE-PAI carries another virulence gene, the *Shigella flexneri* gene *shiA*, whose product has been described to attenuate the host inflammatory response induced by *Shigella flexneri* infections [26]. The observation that SE-PAI is inserted close to the *pheV*-tRNA locus opens the way for speculations on the evolution of *subAB-positive* STEC strains. This locus represents a hot-spot for the integration of PAIs in different bacterial pathogens, such as the *she* PAI of *S. flexneri* 2a [27] and PAI II$_{AL862}$ of the extraintestinal pathogenic *E. coli* strain AL862 [28]. Moreover, this locus represents the insertion site of the LEE PAI in many STEC strains, including those belonging to serotype O103:H2, one of those most commonly associated with severe disease in humans [1]. It could be hypothesized that the LEE-negative, SE-PAI-positive STEC may have arisen from a mutual exclusion event between this PAI and the LEE, which might have competed for the same integration site. This hypothesis could explain the strong association of the *subAB*$_2$ operon with LEE-negative STEC and its apparent absence in LEE-positive strains.

Thus far, little was known about the presence of the two *subAB* allelic variants among STEC. Recently, a study conducted

on different animal species reported a high prevalence of *subAB*$_2$ among LEE-negative strains isolated from small ruminants [14]. In that study, the discrimination between the two allelic variants was based on the intensity of the bands obtained by a non allele-specific PCR amplification of the *subAB* genes, with more intense bands identifying *subAB*$_1$ and faint bands suggesting the presence of *subAB*$_2$ [14]. Another recent study reported that 12 *subAB*-positive O128:H2 STEC strains from different sources were all positive for *tia* [16], suggesting the presence of the *subAB*$_2$-harbouring SE-PAI.

We adopted a PCR-based strategy for the specific detection of the two allelic variants of *subAB* to investigate their presence in a vast collection of *E. coli* strains isolated from human and animal sources. The screening of a panel of strains isolated from cases of human disease and belonging to different pathotypes confirmed that *subAB* genes are mainly confined to STEC lacking the LEE locus. A further screening of a larger collection of LEE-negative STEC strains isolated from patients with diarrhoea showed that the *subAB* genes were present in more than 70% of the isolates, thus supporting the hypothesis that SubAB may play a role in the disease caused by these STEC strains [1]. Interestingly, almost all the *subAB*-positive strains were positive for the *subAB*$_2$ variant, and the concomitant presence of *tia* suggests that the Subtilase-coding genes were carried by SE-PAI.

The high prevalence of the *subAB*$_2$ variant observed in human LEE-negative STEC prompted us to investigate the possible sources of such pathogenic *E. coli*. As the association of *subAB*$_2$ with STEC from small ruminants has been previously suggested [14], we examined a large collection of LEE-negative STEC isolated from sheep by using the same allele-specific PCR strategy. The presence of *subAB* genes was observed in 86% of the strains and, similarly to the human isolates, all the strains possessed the *subAB*$_2$ allelic variant with the exception of two untypeable strains. Again, most *subAB*$_2$-positive strains (84%) were also positive for *tia*, suggesting the presence of SE-PAI.

Altogether, these observations indicate that *subAB*$_2$ represents the prevalent allelic variant in the SubAB-positive, LEE-negative STEC either colonizing sheep or causing diarrhoea in humans, at least among the strains circulating in European countries.

The *subAB*$_2$ operon was also present in one ETEC strain. This finding may be related to the presence of *tia* in SE-PAI. As a matter of fact, the *tia gene* was firstly described as a virulence gene of ETEC [23], suggesting that SE-PAI may have risen as a mosaic structure through recombination events involving ETEC, before being stably acquired by LEE-negative STEC.

The high prevalence of *subAB*$_2$ genes in STEC of ovine origin suggests that sheep may represent an important animal reservoir of *subAB*$_2$-positive, LEE-negative STEC and confirms the zoonotic origin of these human infections. Further studies are needed to (i) assess the role of other ruminant species in the epidemiology of these infections and (ii) elucidate whether *tia* and/or other genes carried by SE-PAI may be involved in the colonization of the host intestinal mucosa by *subAB*$_2$-positive STEC, acting as an alternative to the attaching and effacing machinery.

Transparency Declaration

No conflict of interest to declare.

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