American Society for Microbiology | Applied and Environmental Microbiology

Molecular Profiling of Shiga Toxin-Producing *Escherichia coli* and Enteropathogenic *E. coli* Strains Isolated from French Coastal Environments

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ABSTRACT

Shiga toxin-producing *Escherichia coli* (STEC) and enteropathogenic *E. coli* (EPEC) strains may be responsible for food-borne infections in humans. Twenty-eight STEC and 75 EPEC strains previously isolated from French shellfish-harvesting areas and their watersheds and belonging to 68 distinguishable serotypes were characterized in this study. High-throughput real-time PCR was used to search for the presence of 75 *E. coli* virulence-associated gene targets, and genes encoding Shiga toxin (*stx*) and intimin (*eae*) were subtyped using PCR tests and DNA sequencing, respectively. The results showed a high level of diversity between strains, with 17 unique virulence gene profiles for STEC and 56 for EPEC. Seven STEC and 15 EPEC strains were found to display a large number or a particular combination of genetic markers of virulence and the presence of *stx* and/or *eae* variants, suggesting their potential pathogenicity for humans. Among these, an O26:H11 stx_{1a} *eae*-β1 strain was associated with a large number of virulence-associated genes ($n = 47$), including genes carried on the locus of enterocyte effacement (LEE) or other pathogenicity islands, such as OI-122, OI-71, OI-43/48, OI-50, OI-57, and the high-pathogenicity island (HPI). One O91:H21 STEC strain containing 4 *stx* variants (stx_{1a}, stx_{2a}, stx_{2c}, and stx_{2d}) was found to possess genes associated with pathogenicity islands OI-122, OI-43/48, and OI-15. Among EPEC strains harboring a large number of virulence genes (*n*, 34 to 50), eight belonged to serotype O26:H11, O103:H2, O103:H25, O145:H28, O157:H7, or O153:H2.

IMPORTANCE

The species *E. coli* includes a wide variety of strains, some of which may be responsible for severe infections. This study, a molecular risk assessment study of *E. coli* strains isolated from the coastal environment, was conducted to evaluate the potential risk for shellfish consumers. This report describes the characterization of virulence gene profiles and *stx*/*eae* polymorphisms of *E. coli* isolates and clearly highlights the finding that the majority of strains isolated from coastal environment are potentially weakly pathogenic, while some are likely to be more pathogenic.

Escherichia coli is a commensal aerobic bacterium of the warm-blooded animal intestinal microbiota and is used as a fecal indicator in the environment to classify shellfish-harvesting and bathing areas (1). However, *E. coli* can become pathogenic through the acquisition of mobile genetic elements such as bacteriophages, pathogenicity islands, and plasmids. Among pathogenic *E. coli* strains are the Shiga toxin-producing *Escherichia coli* (STEC) and enteropathogenic *E. coli* (EPEC) strains.

STEC can cause infections ranging from uncomplicated diarrheas to hemorrhagic colitis (HC) and hemolytic-uremic syndrome (HUS). Several STEC serotypes have been involved in numerous food-borne outbreaks worldwide (2, 3), and these strains have been identified as enterohemorrhagic *E. coli* (EHEC). Although *E. coli* O157:H7 has been the main serotype implicated in HC and HUS since the early 1980s, recent studies have shown that non-O157 serotypes are also responsible for numerous human STEC infections. The serogroups most commonly implicated in human STEC infections in the United States are O26, O45, O103, O111, O121, O145, and O157 (4), whereas in Europe, five major EHEC serogroups (the top five; O157, O26, O103, O111, and O145) dominate (5).

The primary virulence factor of STEC is the Shiga toxin, encoded by a lambdoid bacteriophage, which inhibits host cell protein synthesis (6). Within the two major types of Shiga toxin, namely, Stx1 and Stx2, three subtypes of the stx_1 gene (stx_{1a}, stx_{1c}, and stx_{1d}) and seven subtypes of stx_2 gene (stx_{2a}, stx_{2b}, stx_{2c}, stx_{2d}, stx_{2e}, stx_{2f}, and stx_{2g}) have been identified (7). Specific *stx* subtypes are involved in human infections (e.g., stx_{2a}, stx_{2c}, and stx_{2d} are often isolated from patients with HUS) (8, 9), whereas others are related to nonhuman animal infections (e.g., stx_{2e}, causing edema disease in pigs [10]). Certain *stx* subtypes present within a strain may indicate its source: stx_{2c} is prevalent in cattle (11), whereas stx_{2f} is associated mainly with pigeons (12).

The STEC strains implicated in the major cases of human infection (also referred to as "typical EHEC" strains) possess the LEE (locus of enterocyte effacement) pathogenicity island, which is

Received 26 January 2016 **Accepted** 17 April 2016

Accepted manuscript posted online 22 April 2016

Citation Balière C, Rincé A, Delannoy S, Fach P, Gourmelon M. 2016. Molecular profiling of Shiga toxin-producing *Escherichia coli* and enteropathogenic *E. coli* strains isolated from French coastal environments. Appl Environ Microbiol 82:3913–3927. doi:10.1128/AEM.00271-16.

Editor: T. E. Besser, Washington State University

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involved in attaching and effacing (A/E) lesions on intestinal epithelial cells (13). Eighteen types and nine subtypes of the *eae* gene, namely, α, α2, β1 to β3, γ1, γ2, δ, ε, ε2 to ε4, ζ, η, η2, θ, ι, ι2, κ, λ, μ, ν, ξ, ο, π, ρ, and σ, have been deposited in the GenBank database (14). The *eae* subtypes are responsible for some host tissue cell tropisms (15) and are related to human infections; *eae* subtypes β, γ, ε, and θ are those most frequently associated with human infections (16, 17).

However, the emergence of human infections linked to LEE-negative STEC strains indicates that this pathogenicity island is not the only factor responsible for the adherence of bacteria and suggests the presence of other virulence factors carried by other pathogenicity islands or plasmids (18). Additional proteins associated with attachment have been proposed as adhesive factors. For example, Paa is involved in intimate attachment of the bacteria to enterocytes and induced typical A/E lesions in pigs (19). EhaA, an enterohemorrhagic *E. coli* autotransporter, is involved in attachment to biotic and abiotic surfaces (20). Saa is an autoagglutinating adhesin unique to LEE-negative STEC (21). Long polar fimbriae (Lpf) facilitate the attachment of the bacteria to murine Peyer's patches (22).

In addition to genes located on the LEE, a large number of non-LEE effector genes located on other pathogenicity islands (*nleA*, *nleB*, *nleC*, *nleD*, *nleE*, *nleG*, etc.) have been identified in strains responsible for human infections. These genes are involved in various functions, such as the inhibition of phagocytosis, disruption of host innate immune responses, and blockage of cell division (23).

Besides Shiga toxins, other hemolysins or toxins have been identified in the pathogenesis of STEC strains. These include, for example, the enterohemolysin, encoded by the *ehxA* gene, which is linked to cytotoxic effects on endothelial cells (24), the enteroaggregative *E. coli* (EAEC) heat-stable enterotoxin, encoded by the *astA* gene (25), and the alpha-hemolysin, encoded by the *hlyA* gene (26).

Among the virulence factors, the presence of various combinations of type III effector genes, toxin-producing genes, or adhesin-producing genes in pathogenicity islands (OI-122, OI-43/48, OI-57, OI-71, and the high-pathogenicity island [HPI]), on plasmids, or on chromosomes was used to distinguish EHEC from STEC strains and to perform molecular risk assessment based on the presence of several virulence genes in STEC strains (27–31).

Among pathogenic *E. coli* strains, there are also EPEC strains. These are involved in the majority of infantile watery diarrheas in low-income countries but are rarely involved in adult diarrhea (32). EPEC strains are characterized by the presence of the LEE, containing the *eae* gene, as described above. They are classified into typical EPEC and atypical EPEC strains on the basis of the presence of the EPEC adherence factor (EAF) plasmid. The plasmid harbors the *bfp* operon, encoding the bundle-forming pilus, which is involved in the initial adherence of strains to intestinal epithelial cells (32). Twelve serogroups have been recognized as EPEC by the World Health Organization: O26, O55, O86, O111, O114, O119, O125, O126, O127, O128, O142, and O158 (33).

EPEC strains could possess the majority of the virulence genes described above other than *stx* genes. Furthermore, EPEC strains could be lysogenized by *stx*-converting bacteriophages and consequently could become EHEC strains. Conversely, STEC strains possessing the *eae* gene could lose the *stx*-converting bacteriophage and become EPEC or EHEC-like strains (34).

The main reservoir of STEC is cattle (11). However, other animals, such as sheep, goats, swine, birds, wild animals, and humans, can also harbor STEC strains in their digestive tracts (35–37). For typical EPEC, the only reservoir is humans, whereas for atypical EPEC, both humans and other animals can be reservoirs (37). The environment may be contaminated with STEC and EPEC through the spreading of livestock manure on pastures, via wastewaters from slaughterhouses or treatment plant effluents, or by wildlife (38, 39). Only a few studies have focused on the prevalence and description of STEC and EPEC strains in the environment and particularly on the virulence gene profiles of such strains (40–43). Thus, we were prompted to characterize STEC and EPEC strains isolated from French shellfish-harvesting areas.

In the present study, high-throughput microfluidic real-time PCR methods, which had been developed previously and used to investigate the pathogenic potential of *E. coli* strains isolated mainly from animal feces (10), from the carcasses of cattle (44), and from food and animals (45, 46), were used. During a 2-year study, STEC and EPEC strains were isolated from three French shellfish-harvesting areas (shellfish, sediment, and seawater samples) and their watersheds (river water samples), (47). In addition to the *stx* and *eae* genes already investigated, 75 *E. coli* virulence-associated gene targets were examined in these strains using high-throughput microfluidic PCR.

MATERIALS AND METHODS

Bacterial strains and DNA extraction. Most of the strains used in this study were isolated between February 2013 and January 2015 from three French shellfish-harvesting areas and their watersheds, located on the English Channel coast (47). Three strains were isolated from other French shellfish-harvesting areas in 2006 (48). A total of 28 STEC and 75 EPEC strains belonging to 68 distinguishable serotypes isolated from three types of shellfish (oysters, mussels, and cockles) and from freshwater, seawater, and surface sediment samples were investigated. The determination of their serotypes, phylogroups and MLST (multilocus sequence typing) sequence types (STs) has been described previously (47, 48). After cultivation of bacteria on Tryptone Bile X–glucuronide agar (TBX) (AES Chemunex, Bruz, France) at 37°C for 24 h, DNA was extracted with the InstaGene Matrix kit (Bio-Rad, Nanterre, France) according to the manufacturer's instructions.

Characterization of the *stx* subtypes. STEC strains ($n = 28$) were previously identified by Balière et al. (47) by PCR using the *stx* primers and probes described by Perelle et al. (49), and *stx* subtyping was performed here using the PCR tests described by Scheutz et al. (7) for targeting the stx_{1a}, stx_{1c}, and stx_{1d} subtypes and the stx_{2a}, stx_{2b}, stx_{2c}, stx_{2d}, stx_{2e}, stx_{2f}, and stx_{2g} subtypes. The stx_2 subtypes were also determined by sequencing using primers F4, R1, F4-f, and R1-e/f (see Table 1).

Characterization of the *eae* subtypes. *E. coli eae*-positive strains ($n = 76$) were identified by Balière et al. previously (47) with the *eae* primers and probe described by Nielsen and Andersen (50). The *eae* subtypes were determined here by sequencing 311- to 722-bp amplicons of the 3′ variable region of the *eae* gene. Amplicons were obtained with the universal EAE-F and EAE-RB primers, which target the 3′ variable regions of the *eae*-α1, -α2, -β1, -β2, -γ2, -θ, -δ, -κ, -ζ, and -ι subtypes or with primers targeting the 3′ variable regions of the *eae*-ε, -η, -ξ, -μ, and -ν subtypes (Table 1). The amplicons obtained by following the PCR program described by Blanco et al. (51) were subjected to gel electrophoresis and were then sequenced in both directions with the corresponding primers using the fluorescent dye terminator Sanger method (ABI 3730 system; Applied Biosystems) by Eurofins Genomics (Ebersberg, Germany). DNA sequences were edited using the BioEdit program (52) and were compared with the GenBank database. The first valid published sequence with 100% similarity was chosen to identify the *eae* variant.

TABLE 1 PCR primers used in this study for *stx* and *eae* subtyping by sequencing

Gene	Primer designation	Primer sequence (5′–3′)	Fragment size (bp)	Reference or source
stx_2	F4	GGCACTGTCTGAAACTGCTCCTGT	627	7
	R1	ATTAAACTGCACTTCAGCAAATCC		
	F4-f	CGCTGTCTGAGGCATCTCCGCT	625	
	R1-e/f	TAAACTTCACCTGGGCAAAGCC		
eae[a]	EAE-F	ATTACTGAGATTAAGGCTGAT	682	51
	EAE-RB	ATTTATTTGCAGCCCCCCAT		
eae-ε	EAE-F	ATTACTGAGATTAAGGCTGAT	722	51
	LP5	AGCTCACTCGTAGATGACGGCAAGCG		
eae-η	EAE-F	ATTACTGAGATTAAGGCTGAT	712	51
	LP8	TAGATGACGGTAAGCGAC		
eae-ξ	EAE-F	ATTACTGAGATTAAGGCTGAT	468	51
	B49R	ACCACCTTTAGCAGTCAATTTG		
eae-μ	FV373F	CAACGGTAAGTCTCAGACAC	443	51
	FV373R	CATAATAAGCTTTTTGGCCTACC		
eae-ν	IH1229aF	CACAGCTTACAATTGATAACA	311	51
	IH1229aR	CTCACTATAAGTCATACGACT		
eae[b]	eae-F1	ACTCCGATTCCTCTGGTGAC	~1,800–2,100, depending on the allele	75
	escD-R1	GTATCAACATCTCCCGCCCA		
eae[b]	cesT-F3	CAGGAGCACAATCGCTGTTG	1,727	This study
	eae-R3	CAGACGATACGATCCAGACC		

[a] Universal primers targeting the 3′ variable regions of the *eae*-α1, -α2, -β1, -β2, -γ2, -θ, -δ, -κ, -ζ, and -ι subtypes.
[b] Primers targeting overlapping DNA fragments of the *eae* gene.

Nine *E. coli* strains, which were PCR negative for these primer combinations, were chosen for sequencing of the entire *eae* gene. Two overlapping DNA fragments of the *eae* gene were PCR amplified using primers eae-F1 and escD-R1 or primers cesT-F3 and eae-R3, respectively (Table 1). The PCR program included a 10-min initial denaturing step at 94°C, followed by 35 cycles of amplification (94°C for 20 s, 56°C for 20 s, and 72°C for 90 s) and a final extension step at 72°C for 5 min. The PCR amplicons were then subjected to gel electrophoresis and were purified using the NucleoSpin Extract II kit (Macherey-Nagel, Düren, Germany) before being sequenced as described above.

High-throughput real-time PCR system. The BioMark real-time PCR system (Fluidigm, San Francisco, CA) was used for high-throughput microfluidic real-time PCR amplification using the 96.96 dynamic arrays (Fluidigm). Amplifications were performed in accordance with the recommendations of the manufacturer, using the EvaGreen DNA binding dye (Biotium Inc., Hayward, CA) followed by melting curve analysis. The BioMark real-time PCR system was used with the following thermal profile: 95°C for 10 min (enzyme activation), followed by 35 cycles of 95°C for 15 s and 60°C for 1 min (annealing of primers and amplification step).

Seventy-five *E. coli* virulence-associated gene targets were selected according to their roles in pathogenesis, their association with human and nonhuman animal illness, and their usefulness, as demonstrated previously, for the characterization of STEC and EPEC strains, isolated mainly from finishing swine (10), human patient and animal feces, food, and animals (45, 46), the carcasses of cattle originating from different farms and food (44), and the feces of adults and children (28, 30, 31, 43).

Virulence gene targets were classified into five groups according to function: the adhesion group (*eibG*, *iha*, *saa*, *toxB*, *orfA*, *orfB*, *paa*, *stcE*, *sab*, *efa1* or *lifA*, *bfpA*, *espP*, the F6/F987P [*fasA*], F18/F107 [*fedA*], and F41 [*fimF41a*] genes, $lpfA_{O157}$, $lpfA_{O26}$, $lpfA_{O113}$, *ehaA*, and *epeA* [$n = 20$]), the type III secretion system group (*espB*, *espD*, *espA*, *tir*, *espZ*, *escC*, *espG*, *escD*, *escN*, *escV*, *escJ*, *nleB*, *nleE*, *nleF*, *nleG*, *nleH1-2*, *nleA*, *espM1*, *nleC*, *nleD*, *nleH1-1*, *nleG2*, *nleG5-1*, *nleG5-2*, *nleG6-2*, *espV*, *espK*, *espN*, *espJ*, and *espM2* [$n = 30$]), the toxin group (*subA*, *ent*, *ehxA*, *cdt-I*, *cdt-III*, *cdt-V*, *sta*, *lt*, *hlyA*, *cnf1*, *cnf2*, *astA* [$n = 12$]), the resistance and persistence group (*katP*, *ecf1*, *pagC*, *terE*, and *ureD* [$n = 5$]), and the "other function" group (*aggR*, *pic*, *irp2*, *fyuA*, Z2098, ECs1763, ECs1822, and *etpD* [$n = 8$]). The *wecA* gene was used as an *E. coli* reference genetic marker.

RESULTS

STEC and EHEC virulence gene profiles. A collection of *E. coli* strains, comprising 27 STEC (*stx*-positive, *eae*-negative) strains and 1 EHEC (*stx*-positive, *eae*-positive) strain collected from French coastal areas, was investigated. Eleven STEC strains were positive for the stx_1 gene only, and 13 were positive for the stx_2 gene only, while 4 strains harbored a combination of the stx_1 and stx_2 genes. The most commonly identified stx_1 subtype was stx_{1d} (23% of *stx* genes detected), followed by stx_{1a} (17%) (Fig. 1A; Table 2). The most common stx_2 subtype was stx_{2a} (17%) (Fig. 1A). Five strains were shown to possess several *stx* subtypes: three possessed stx_{1a} and stx_{2a} and belonged to serotypes O185:H28, ONT:H11, and O130:H11; one possessed stx_{2a} and stx_{2d}, and one possessed stx_{1a}, stx_{2a}, stx_{2c}, and stx_{2d}, corresponding to serotypes O8:H19 and O91:H21, respectively (Table 2). Three strains harbored either the stx_{1c}, stx_{2e}, or stx_{2g} variant. Six strains harbored at least one of the stx_{2a}, stx_{2c}, and stx_{2d} variants, which had been reported previously (8, 9) as being often isolated from patients with HUS (Table 2).

Among the STEC strains investigated, 17 unique virulence gene profiles were identified, based on virulence gene detection

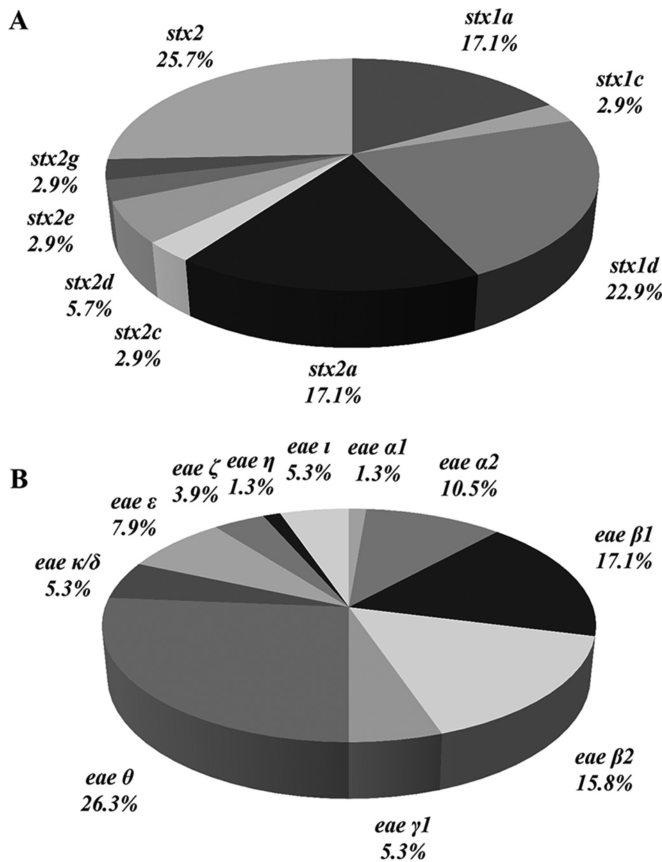

FIG 1 Subtyping of genes encoding Shiga toxin and intimin. (A) Distribution of *stx* gene subtypes found in STEC strains. (B) Distribution of *eae* gene subtypes found in *E. coli* strains.

(Table 2). The EHEC O26:H11 strain harbored the profile with the highest number of *E. coli* virulence-associated gene targets, with 47 genes detected (profile 1). In addition to the genes carried on the LEE and linked to the type III secretion system and to the *eae* gene (and specifically the *eae*-β1 subtype [Table 2]), this strain harbors genes associated with pathogenicity islands such as OI-122 (*nleB*, *nleE*, and *ent*), OI-71 (*espM1*), OI-43/48 (*ureD* and *terE*), OI-50 (*espK* and *espN*), OI-57 (Z2098), and HPI (*irp2* and *fuyA*). One strain of serotype O91:H21 (profile 2) was isolated and was found to possess genes associated with certain pathogenicity islands, such as OI-122 (*pagC*), OI-43/48 (*iha*), and OI-15 (*ehaA*), and with plasmids (pO113 [*sab* and *epeA*] and pO157 [*espP* and *ehxA*]). For the other STEC strains, 2 to 12 genes were detected, corresponding to 15 additional virulence gene profiles. These strains did not possess genes from the LEE, or genes associated with pathogenicity island OI-122, OI-71, OI-50, or HPI. Serotype O100:HNM, the serotype most commonly represented in isolated STEC strains ($n = 9$), was associated with only two additional virulence genes (Table 2).

In those STEC strains without the LEE pathogenicity island, other genes involved in adhesion were detected. Of these, the *paa* gene, which encodes the porcine A/E-associated gene, was detected most frequently (in 57% of STEC strains) (Fig. 2 and 3A). This was followed by the *ehaA* gene, which was detected in 54% of the strains. The other genes involved in adhesion that were detected were mainly those encoding long polar fimbriae ($lpfA_{O113}$ [in 39% of strains] and $lpfA_{O26}$ [18%]), extracellular serine protease (*espP*) (32%), the IrgA homologue adhesin (*iha*) (21%), an autoagglutinating adhesin (*saa*) (18%), and an autotransporter (*sab*) (18%).

In addition to the *stx* genes, genes that could lead to the production of other toxins or hemolysins were present in 11 different STEC strains (corresponding to 11 different virulence profiles). The most frequent toxin-associated gene detected was *ehxA* (in 25% of strains) (Fig. 2). The *subA*, *cdt-V*, and *astA* genes, encoding the subtilase cytotoxin, a cytolethal distending toxin, and an EAEC heat-stable enterotoxin, respectively, were each detected in 11% of the strains. The *sta* gene, encoding a heat-stable toxin, and the *hlyA* gene, encoding the alpha hemolysin, were both detected in 7% of the strains.

EPEC virulence gene profiles. Seventy-five EPEC strains (i.e., *eae*-positive and *stx*-negative strains) from the French coastal areas were also investigated. The EPEC strains isolated were mainly atypical EPEC strains (93%), since the typical EPEC marker *bfpA* was detected in only 7% of these strains (Fig. 3B).

The most commonly identified *eae* subtype was *eae*-θ (26% of strains), followed by *eae*-β1 (17%), *eae*-β2 (16%), and *eae*-α2 (11%) (Fig. 1B). All four O26:H11 EPEC strains isolated from shellfish and freshwater harbored *eae*-β1 (Table 2). All the strains of serotypes O63:H6/HNM ($n = 5$) and O125:H6 ($n = 2$) harbored *eae*-α2, even though they belonged to four different profiles (profiles 30, 32, 37, and 43). The same observation was made for strains of serotypes O33:H6 ($n = 2$) and O113:H6 ($n = 4$), which belonged to six different profiles but all harbored the *eae*-β2 subtype, and for strains of serotypes O23:H8 ($n = 2$) and ONT:H8 ($n = 2$), which belonged to four different profiles but all harbored the *eae*-θ subtype. The other 21 strains harbored one of the following *eae* subtypes: ε, γ1, κ/δ, ι, ζ, or η (Table 2).

Among the 75 EPEC strains, 56 unique virulence gene profiles were identified, based on the virulence genes detected, suggesting a high diversity of virulence genes in the EPEC strains isolated from the environment (Table 2; Fig. 3B). The number of virulence genes detected ranged from 11 to 50. Most of the profiles were represented by one serotype, except for seven EPEC profiles (profiles 3, 13, 37, 42, 45, 52, and 53) that were represented by two to four different serotypes. EPEC profile 37, corresponding to serotypes O63:H6/HNM and ONT:H6, with 17 virulence genes, was the profile most commonly identified among EPEC strains ($n = 5$ [Table 2]). The profiles with the highest numbers of *E. coli* virulence-associated genes were represented by 10 strains that belonged to the top five EHEC serotypes (i.e., O26:H11, O103:H2, O103:H25, O145:H28, and O157:H7) and to serotype O153:H2, harboring 34 to 50 virulence genes. These strains possess genes carried by some pathogenicity islands, such as OI-122, OI-71, and OI-43/48 (Table 2), and at least one of the genes *espM1*, *espK*, *espV*, *espN*, *ureD*, and Z2098, which are highly associated with typical EHEC (Fig. 3B). The latter genes were also detected in 13 additional EPEC strains corresponding to serotypes O125:H6, O128:H2, O159:H7, O167:H3, O23:H8, O28:HNM, O29:H19, O40:HNM, O71:HNM, O86:H31, O88:H8, and O98:H8 (Fig. 3B).

In addition to *eae*, seven genes (*espB*, *espD*, *escC*, *espG*, *escD*, *escV*, and *escJ*), all of which were carried by the LEE, were detected in 100% of EPEC strains. The other genes that were detected most frequently (i.e., *espM2*, *escN*, *nleC*, *nleH1-2*, *espJ*, and *nleH1-1*) were observed in >60% of strains and are associated with the type III secretion system (Fig. 2). Among the 20 genes of the adhesion

group that were investigated, *paa* was detected most frequently (in 60% of strains), followed by *ehaA*, $lpfA_{O113}$, and $lpfA_{O26}$, found in 39%, 33%, and 29% of strains, respectively, and by the *efa1* or *lifA* gene, encoding an EHEC factor for adherence (20%) (Fig. 2). A number of genes that encode toxins and hemolysins were present in EPEC strains. For example, 33% of strains carried the *astA* gene, which encodes the EAEC heat-stable toxin. The *ent* gene, which encodes an ankyrin repeat, was also detected in 33% of the EPEC strains. The *ehxA* gene, which encodes an enterohemolysin, and *cdt-I*, which encodes a cytolethal distending toxin, were detected in 11% and 7% of EPEC strains, respectively. Finally, the *hlyA* gene, which encodes alpha-hemolysin, was present in 5% of EPEC strains.

Genes not detected in STEC and EPEC strains. Nine virulence genes were never detected in STEC and EPEC strains. Four of these are linked to the adhesion function (*eibG* and the F6/F987P [*fasA*], F18/F107 [*fedA*], and F41 [*fimF41a*] genes), four to the toxin production function (*cdt-III*, *lt* [*elt*], *cnf1*, and *cnf2*), and one (*aggR*) to the enteroaggregative function. No obvious association was observed between the virulence profiles and the types of samples from which the strains were isolated (shellfish, water, or sediment).

DISCUSSION

This study presents a molecular risk assessment of STEC and EPEC strains isolated in France from shellfish, seawater, and sediment samples collected in shellfish-harvesting areas and from freshwater samples in their upstream watersheds. These strains derived from a larger collection of *E. coli* strains (12,016 isolates) isolated during a recent study (47). The molecular risk assessment of 28 STEC and 75 EPEC strains was conducted by testing a large panel of virulence genetic markers (i.e., a total of 75 markers) associated with human and animal infections using a high-throughput real-time PCR approach and by identifying *stx* and *eae* subtypes.

Some STEC and EPEC strains characterized in this study were found to display a large number or a particular combination of virulence genetic markers and the presence of *stx* and/or *eae* variants, suggesting their potential pathogenicity for humans. The identification of *E. coli* strains that pose a significant threat to human health is still challenging and requires the screening of more genetic markers than only *stx* and *eae* genes. The *stx* and *eae* genes are, respectively, hallmarks of STEC (including pathogenic and nonpathogenic strains) and EPEC strains, but the genetic basis of *E. coli* pathogenicity is much more complex than the presence or absence of one or both of these genes. The literature reports many genetic markers that may play roles in the virulence of *E. coli* and some variants of the *eae* and/or *stx* genes (for example) that are closely associated with human-pathogenic *E. coli* strains. In this study, we took this information into account to define criteria that, according to the current literature, could best recapitulate the most virulent strains isolated from human patients.

Among the *stx*-positive strains, only the EHEC O26:H11 stx_{1a} *eae*-β1 strain was associated with a large number of virulence-associated gene targets (60% of genes). Furthermore, this strain, isolated from shellfish, was the only one to harbor the two EHEC gene markers *stx* and *eae*. The combined presence of *stx*, *eae*, and the 45 supplementary virulence genes has been associated with enhanced virulence. Similar O26:H11 stx_{1a} *eae*-β1 strains (sequence type 21; phylogroup B1) have been isolated previously from human patients with HUS or diarrhea in Europe (53, 54).

Although they lack the *eae* gene, most of the STEC strains isolated here have the genetic potential to adhere to host cells through other structures. For example, 18% of *eae*-negative STEC strains displayed the *saa* gene, encoding the STEC autoagglutinating adhesion factor. This gene was observed only in LEE-negative STEC strains, in agreement with previous findings from strains of human and bovine origins (55). Other genes encoding proteins associated with attachment were detected in some strains. These include *paa*, detected here in 15 *eae*-negative STEC strains, as well as *ehaA*, $lpfA_{O113}$, $lpfA_{O26}$, *espP*, *iha*, and *sab*. Many of these genes have been detected in *eae*-negative strains isolated previously, in other studies, and could play a role in adhesion to host cells and consequently in the virulence potential of the isolated strains (19–22, 56).

Among the strains that were negative for the *eae* gene, a STEC strain of serotype O91:H21, isolated from freshwater, could potentially be pathogenic for humans as a result of (i) the presence of seven alternative adhesion factors (*saa*, *ehaA*, $lpfA_{O113}$, $lpfA_{O26}$, *espP*, *iha*, and *sab*), some of which are included in pathogenicity islands such as OI-15 or OI-43/48, or (ii) the presence of the three stx_2 variants stx_{2a}, stx_{2c}, and stx_{2d}. Furthermore, this strain also possesses the *cdt-V* gene, encoding a toxin, which has been found in STEC strains involved in serious diseases (57). Finally, this strain presented sequence type ST442, which has been found to be the unique ST associated with hemolytic-uremic syndrome among the 10 STs identified in 100 STEC O91 strains isolated from different patients (58).

In addition to the O91:H21 strain, five other STEC strains harbored at least one of the stx_{2a}, stx_{2c}, or stx_{2d} variants, and four of them (i.e., O8:H19, O185:H28, ONT:H11, and O130:H11) were found to combine several distinct stx_1 and/or stx_2 subtypes. The presence of a combination of *stx* genes has been observed previously among strains of similar serotypes isolated from humans (18). Furthermore, in the study of Bertin et al. (59), strains harboring two or three *stx* subtypes were found to be highly cytotoxic toward Vero cells more frequently than other strains. We can hypothesize that strains with a combination of stx_1 and/or stx_2 subtypes are more virulent than others. Furthermore, strains of serotypes O8:H19 and O130:H11 were isolated from human patients previously (60). The latter strain, like the O91:H21 strain described above, harbors the *iha*, $lpfA_{O113}$, *ehxA*, and *cdt-V* genes, suggesting a potential virulence trait for humans. Indeed, these genetic markers have already been identified in human LEE-negative STEC strains associated with diseases (18, 61).

Subtypes stx_{1a} and stx_{2a} were each found in 17% of the STEC strains (essentially from freshwater samples) and have been associated with only six to eight supplementary virulence genes. These subtypes have frequently been identified in STEC strains from human, animal, environmental, and food samples (62–65). STEC strains with stx_{2a} have been found to be associated with several clinical symptoms, such as HUS and HC, whereas STEC strains with stx_{1a} have been associated mainly with diarrhea without HUS (8).

On the other hand, the simultaneous detection of subtype stx_{2e} and the *paa*, *orfA*, *hlyA*, and ECs1763 genes in the serotype O2:H32 strain (from a seawater sample) suggests that this strain could potentially be associated with swine edema disease (10) or could derive from a pig source (64). In the same way, strain O15:H16

TABLE 2 Distribution of STEC and EPEC strains by serotype and virulence gene profiles[a,b]

Strain no.	Serotype[a]	Sample[b] Type	Site	Date	Origin	*E. coli* pathogroup	Virulence gene subtype stx_1	stx_2	*eae*	Profile no.	Total no. of genes detected	No. of genetic markers in the following group[c]: Adhesion ($n = 20$)	Type III secretion system ($n = 30$)	Toxin ($n = 12$)	Other function ($n = 13$)	OI-71 ($n = 5$)	OI-122 ($n = 5$)	OI-57 ($n = 6$)	OI-43/48 ($n = 3$)	HPI ($n = 2$)	EHEC markers ($n = 4$)	*nle* genes ($n = 13$)	Phylogroup[a,d]	ST[a] (no. of strains)
78	O26:H11	Shellfish	1	November 2013	M1.2	STEC	1a	−	β1	1	47	8	25	3	9	5	4	6	3	2	4	12	B1	21
95	O91:H21	Freshwater	3	May 2013	W3.2	STEC	1a	2a, 2c, 2d	−	2	15	8	0	2	1	0	1	0	1	0	0	0	B1	442
59	O130:H11	Freshwater	1	December 2013	W1.1	STEC	1a	2a	−	3	12	7	1	2	0	0	0	0	1	0	0	0	B1	ND
14	ONT:H11	Freshwater	1	May 2013	W1.1	STEC	1a	2a	−	4	11	7	0	2	0	0	0	0	1	0	0	0	B1	295
163	O63:H6	Freshwater	1	November 2014	W1.1	STEC	−	2a	−	5	10	7	0	2	0	0	0	0	1	0	0	0	B1	2105
124	ONT:H10	Freshwater	2	April 2014	W2.1	STEC	1a	−	−	6	9	4	0	3	1	0	0	0	0	0	0	0	B1	1258
88	O185:H28	Freshwater	2	April 2013	W2.1	STEC	1a	2a	−	7	8	5	0	1	0	0	0	0	1	0	0	0	B2	658
72	O2:H32	Seawater	1	February 2014	W1.5	STEC	−	2e	−	8	6	2	0	1	2	0	0	1	1	0	0	0	A	10
126	O8:H19	Freshwater	3	July 2014	W3.1	STEC	−	2a, 2d	−	9	5	3	0	0	0	0	0	0	0	0	0	0	B1	162
16	O154:H31	Shellfish	1	May 2013	O1.1	STEC	1d	−	−	10	5	3	0	0	1	0	0	0	0	0	0	0	D	1892
19	O15:H16	Shellfish	1	June 2013	M1.1	STEC	−	2g	−	11	5	2	0	2	0	0	0	0	0	0	0	0	A	325
127	O76:H19	Freshwater	2	August 2014	W2.3	STEC	1d	−	−	12	5	2	0	2	0	0	0	0	0	0	0	0	B1	675
LR2	O100:H21	Shellfish	6	January 2003	M6.1	STEC	1d	−	−	13	4	3	0	0	0	0	0	0	0	0	0	0	ND	ND
15	O154:H31	Freshwater	1	May 2013	W1.1	STEC	1d	−	−	14	4	3	0	0	0	0	0	0	0	0	0	0	D	1892
44	O154:HNM	Shellfish	1	November 2013	M1.1	STEC	1d	−	−	14	4	3	0	0	0	0	0	0	0	0	0	0	D	1892
20	O100:HNM	Shellfish	2	May 2013	M2.1	STEC	−	+	−	15	3	1	0	0	1	0	0	0	1	0	0	0	A	993
24	O100:HNM	Freshwater	3	May 2013	W3.1	STEC	−	+	−	15	3	1	0	0	1	0	0	0	1	0	0	0	A	993
31	O100:HNM	Shellfish	3	May 2013	O3.1	STEC	−	+	−	15	3	1	0	0	1	0	0	0	1	0	0	0	A	993
32	O100:HNM	Shellfish	3	May 2013	M3.1	STEC	−	+	−	15	3	1	0	0	1	0	0	0	1	0	0	0	A	993
33	O100:HNM	Shellfish	2	May 2013	C2.1	STEC	−	+	−	15	3	1	0	0	1	0	0	0	1	0	0	0	A	993
35	O100:HNM	Shellfish	3	June 2013	M3.1	STEC	−	+	−	15	3	1	0	0	1	0	0	0	1	0	0	0	A	993
36	O100:HNM	Sediment	3	June 2013	S3.1	STEC	−	+	−	15	3	1	0	0	1	0	0	0	1	0	0	0	A	993
122	O100:HNM	Shellfish	2	March 2014	M2.1	STEC	−	+	−	15	3	1	0	0	1	0	0	0	1	0	0	0	A	993
125	O100:HNM	Shellfish	3	June 2014	O3.1	STEC	−	+	−	15	3	1	0	0	1	0	0	0	1	0	0	0	A	993
PB4	O38:H26	Shellfish	5	July 2003	M5.1	STEC	1c	−	−	16	3	1	0	1	0	0	0	0	0	0	0	0	ND	ND
1	O149:H1	Freshwater	1	April 2013	W1.1	STEC	1d	−	−	17	2	1	0	0	0	0	0	0	0	0	0	0	D	132
3	O149:HNM	Freshwater	1	April 2013	W1.1	STEC	1d	−	−	17	2	1	0	0	0	0	0	0	0	0	0	0	D	132
73	O28:H1	Shellfish	1	March 2014	C1.1	STEC	1d	−	−	17	2	1	0	0	0	0	0	0	0	0	0	0	D	132
SP5	O157:H7	Shellfish	4	September 2003	O4.1	EPEC	−	−	γ1	1	50	8	30	3	8	5	5	5	3	0	3	13	ND	ND
77	O145:H28	Shellfish	2	June 2013	C2.1	EPEC	−	−	γ1	2	39	4	24	3	7	3	5	2	3	0	3	7	D	ND
79	O145:H28	Shellfish	2	June 2013	M2.1	EPEC	−	−	γ1	2	39	4	24	3	7	3	5	2	3	0	3	7	D	ND
121	O103:H25	Freshwater	3	March 2014	W3.1	EPEC	−	−	θ	2	35	5	19	3	7	3	5	3	2	0	4	7	B1	343
96	O103:H2	Freshwater	2	June 2013	W2.3	EPEC	−	−	θ	3	35	5	19	3	7	3	5	3	2	0	4	7	B1	343
120	O153:H2	Freshwater	2	March 2014	W2.3	EPEC	−	−	ε	4	34	5	19	2	7	1	5	2	2	2	2	8	B1	27
123	O153:H2	Shellfish	3	March 2014	M3.1	EPEC	−	−	ε	4	34	5	19	2	7	1	5	2	2	2	2	8	B1	27
81	O26:H11	Shellfish	1	November 2013	M1.2	EPEC	−	−	β1	5	34	5	20	3	5	5	4	3	1	2	0	10	B1	29
84	O26:H11	Freshwater	1	November 2013	W1.3	EPEC	−	−	β1	5	34	5	20	3	5	5	4	3	1	2	0	10	B1	29

85	O26:H11	Shellfish	1	November 2013	C1.1	EPEC	–	–	β1	5	34	5	20	3	5	5	4	3	1	2	0	10	B1	29
102	O23:H8	Freshwater	2	August 2013	W2.1	EPEC	–	–	θ	6	33	5	22	2	3	5	5	5	0	0	0	11	B1	327
129	O23:H8	Shellfish	2	August 2014	C2.1	EPEC	–	–	θ	7	32	5	22	2	2	5	4	5	0	0	0	11	B1	327
80	O26:H11	Freshwater	2	August 2013	W2.1	EPEC	–	–	β1	8	27	4	17	2	3	2	4	2	1	2	0	8	B1	29
64	O39:HNM	Freshwater	1	January 2014	W1.3	EPEC	–	–	ι	9	25	3	19	1	1	3	0	5	0	0	0	10	B1	39
111	ONT:H31	Freshwater	1	October 2014	W1.3	EPEC	–	–	θ	10	24	3	16	3	1	1	3	2	0	0	0	6	B1	40
131	O15:H2	Freshwater	3	August 2014	W3.2	EPEC	–	–	β1	11	23	3	17	1	1	2	5	2	0	0	0	8	B1	20
153	O28:HNM	Freshwater	1	June 2014	W1.1	EPEC	–	–	γ1	12	23	0	15	2	5	1	0	2	2	0	3	2	D	137
61	O153:H21	Freshwater	1	January 2014	W1.1	EPEC	–	–	θ	13	22	3	17	1	0	2	3	1	0	0	0	7	B1	40
115	O108:H21	Freshwater	3	November 2014	W3.1	EPEC	–	–	θ	13	22	3	17	1	0	2	3	1	0	0	0	7	B1	337
117	O108:H21	Shellfish	3	November 2014	W3.2	EPEC	–	–	θ	13	22	3	17	1	0	2	3	1	0	0	0	7	B1	337
118	O108:H21	Shellfish	2	January 2014	C2.1	EPEC	–	–	θ	13	22	3	17	1	0	2	3	1	0	0	0	7	B1	337
134	O146:H21	Shellfish	3	August 2014	M3.1	EPEC	–	–	θ	14	22	3	17	1	0	2	3	1	0	0	0	7	B1	442
65	O40:HNM	Freshwater	1	February 2014	W1.1	EPEC	–	–	β1	15	22	0	16	1	4	3	0	1	1	2	2	6	A	10
162	ONT:H8	Freshwater	1	August 2014	W1.2	EPEC	–	–	θ	16	21	4	15	1	0	1	3	1	0	0	0	5	B1	327
49	O98:H8	Shellfish	1	November 2014	M1.1	EPEC	–	–	ι	17	21	5	15	0	0	1	1	0	0	0	1	5	B1	590
159	O91:H10	Shellfish	1	July 2014	C1.1	EPEC	–	–	β2	18	21	3	14	1	2	1	0	1	0	2	0	4	B2	641
139	O88:H25	Shellfish	3	September 2014	M3.1	EPEC	–	–	β1	19	21	4	15	1	0	1	4	1	0	0	0	6	B1	328
154	ONT:H8	Freshwater	1	April 2014	W1.2	EPEC	–	–	θ	20	20	3	15	1	0	1	3	1	0	0	0	5	B1	327
105	O71:HNM	Freshwater	1	August 2014	W1.2	EPEC	–	–	η	21	20	5	14	0	0	2	0	0	0	0	1	5	A	517
133	O88:H8	Shellfish	3	August 2014	O3.1	EPEC	–	–	ι	22	20	2	17	0	0	3	0	1	0	0	1	7	B1	590
136	O146:H6	Shellfish	2	September 2014	C2.1	EPEC	–	–	θ	23	20	3	15	1	0	1	3	1	0	0	0	5	B1	442
37	O167:H3	Freshwater	1	August 2013	W1.1	EPEC	–	–	β1	24	20	0	17	0	2	5	0	2	0	0	0	7	D	2558
113	O128:H2	Freshwater	2	November 2014	W2.3	EPEC	–	–	β1	25	19	3	13	0	2	1	0	0	0	2	1	3	B1	20
132	O113:H6	Shellfish	3	August 2014	O3.1	EPEC	–	–	β2	26	19	0	14	2	2	1	0	1	0	2	0	4	B2	121
160	ONT:H6	Freshwater	1	August 2014	W1.1	EPEC	–	–	β2	27	19	1	15	0	2	2	0	1	0	2	0	5	B2	28
86	O5:H40	Shellfish	2	February 2014	M2.1	EPEC	–	–	θ	28	19	1	17	0	0	3	2	0	0	0	0	7	A	10
128	O86:H31	Shellfish	2	August 2014	C2.1	EPEC	–	–	θ	29	19	3	14	1	0	2	3	0	0	0	1	4	D	2569
135	O125:H6	Freshwater	2	September 2014	W2.3	EPEC	–	–	α2	30	19	1	15	2	0	2	0	0	0	0	1	4	B2	583
152	ONT:H6	Seawater	1	December 2014	W1.5	EPEC	–	–	β2	31	18	1	13	0	3	1	0	1	1	2	0	4	B2	28

(Continued on following page)

TABLE 2 (Continued)

Strain no.	Serotype[a]	Sample[b] Type	Site	Date	Origin	*E. coli* pathogroup	Virulence gene subtype stx_1	stx_2	*eae*	Profile no.	Total no. of genes detected	No. of genetic markers in the following group[c]: Adhesion ($n = 20$)	Type III secretion system ($n = 30$)	Toxin ($n = 12$)	Other function ($n = 13$)	OI-71 ($n = 5$)	OI-122 ($n = 5$)	OI-57 ($n = 6$)	OI-43/48 ($n = 3$)	HPI ($n = 2$)	EHEC markers ($n = 4$)	*nle* genes ($n = 13$)	Phylogroup[a,d]	ST[a] (no. of strains)
54	O125:H6	Freshwater	1	November 2014	W1.3	EPEC	–	–	α2	32	18	1	15	1	0	2	0	0	0	0	0	4	B2	583
116	O113:H6	Freshwater	3	November 2014	W3.2	EPEC	–	–	β2	33	18	0	14	1	2	1	0	1	0	2	0	4	B2	121
138	ONT:H6	Freshwater	3	September 2014	W3.2	EPEC	–	–	β2	34	18	1	14	0	2	2	0	1	0	2	0	5	B2	28
149	O159:H7	Freshwater	1	November 2014	W1.3	EPEC	–	–	ε	35	18	3	14	0	0	2	0	0	0	0	1	4	B1	ND
104	O157:H16	Freshwater	1	July 2014	W1.4	EPEC	–	–	ε	36	18	0	17	0	0	3	0	4	0	0	0	10	A	10
58	O63:H6	Freshwater	2	November 2014	W2.1	EPEC	–	–	α2	37	17	1	14	0	1	1	0	1	1	0	0	3	B2	122
62	O63:H6	Freshwater	1	January 2014	W1.2	EPEC	–	–	α2	37	17	1	14	0	1	1	0	1	1	0	0	3	B2	122
142	O63:H6	Freshwater	3	October 2014	W3.2	EPEC	–	–	α2	37	17	1	14	0	1	1	0	1	1	0	0	3	B2	122
144	O63:HNM	Freshwater	3	October 2014	W3.2	EPEC	–	–	α2	37	17	1	14	0	1	1	0	1	1	0	0	3	B2	122
141	ONT:H6	Freshwater	3	October 2014	W3.2	EPEC	–	–	α2	37	17	1	14	0	1	1	0	1	1	0	0	3	B2	122
43	O51:HNM	Freshwater	2	October 2013	W2.2	EPEC	–	–	α1	38	17	1	14	0	1	1	0	1	1	0	0	4	B2	589
51	O113:H6	Shellfish	1	November 2013	M1.2	EPEC	–	–	β2	39	17	0	14	0	2	1	0	1	0	2	0	4	B2	121
60	O2:H45	Freshwater	2	December 2013	W2.1	EPEC	–	–	κ/δ	40	17	2	13	1	0	2	0	0	0	0	0	3	B2	1907
63	O179:H31	Freshwater	1	January 2014	W1.2	EPEC	–	–	ζ	41	17	1	13	0	2	1	0	0	0	2	0	4	B2	1092
107	O25:H2	Freshwater	1	September 2014	W1.1	EPEC	–	–	β1	42	17	2	14	0	0	1	0	2	0	0	0	5	B1	20
38	ONT:H2	Freshwater	1	August 2013	W1.2	EPEC	–	–	β1	42	17	2	14	0	0	1	0	2	0	0	0	5	B1	20
47	O63:H6	Freshwater	1	November 2013	W1.4	EPEC	–	–	α2	43	17	1	13	2	0	1	0	0	0	0	0	3	B2	583
42	O29:H19	Freshwater	2	October 2013	W2.1	EPEC	–	–	ε	44	16	3	12	0	0	1	0	0	0	0	1	2	B1	517
46	O116:H20	Freshwater	1	November 2013	W1.3	EPEC	–	–	β1	45	16	1	14	0	0	2	0	0	0	0	0	5	A	10
50	O28:H16	Shellfish	1	November 2013	M1.1	EPEC	–	–	β1	45	16	1	14	0	0	2	0	0	0	0	0	5	A	10
52	O113:H6	Shellfish	1	November 2013	C1.1	EPEC	–	–	β2	46	16	0	13	0	2	1	0	1	0	2	0	3	B2	1583
161	O137:H6	Freshwater	1	August 2014	W1.1	EPEC	–	–	β2	47	16	1	12	0	2	1	0	0	0	2	0	4	B2	2678
39	ONT:H6	Freshwater	1	September 2013	W1.3	EPEC	–	–	β2	48	16	1	12	0	2	1	0	1	0	2	0	4	B2	28
67	O33:H6	Freshwater	1	February 2014	W1.3	EPEC	–	–	β2	49	15	1	10	0	3	0	0	0	1	2	0	0	B2	28
112	O85:H31	Freshwater	1	October 2014	W1.3	EPEC	–	–	ζ	50	13	0	10	0	2	0	0	0	0	2	0	1	B2	803
71	O33:H6	Freshwater	1	February 2014	W1.3	EPEC	–	–	β2	51	13	0	10	0	2	0	0	0	0	2	0	0	B2	28

158	O20:HNT	Seawater	1	April 2014	W1.5	EPEC	–	–	θ	52	12	0	10	1	0	0	0	0	0	0	0	0	A	3747
164	O98:H56	Freshwater	1	December 2014	W1.4	EPEC	–	–	θ	52	12	0	10	1	0	0	0	0	0	0	0	0	A	ND
70	O98:HNM	Seawater	1	February 2014	W1.5	EPEC	–	–	θ	52	12	0	10	1	0	0	0	0	0	0	0	0	A	3747
150	O98:HNT	Freshwater	1	December 2014	W1.4	EPEC	–	–	θ	52	12	0	10	1	0	0	0	0	0	0	0	0	A	3747
40	O71:H49	Shellfish	1	October 2013	C1.1	EPEC	–	–	κ/δ	53	12	0	11	0	0	0	0	0	0	0	0	2	B2	2813
41	O71:H49	Shellfish	1	October 2013	M1.2	EPEC	–	–	κ/δ	53	12	0	11	0	0	0	0	0	0	0	0	2	B2	2813
48	O8:H14	Freshwater	1	November 2013	W1.4	EPEC	–	–	κ/δ	53	12	0	11	0	0	0	0	0	0	0	0	2	B2	4178
106	O85:HNM	Freshwater	1	August 2014	W1.4	EPEC	–	–	ζ	54	12	0	9	0	2	0	0	0	0	2	0	1	B2	803
74	O98:HNM	Freshwater	1	March 2014	W1.4	EPEC	–	–	θ	55	11	0	9	1	0	0	0	0	0	0	0	0	A	3747
103	O145:H34	Freshwater	1	July 2014	W1.3	EPEC	–	–	ι	56	11	0	10	0	0	0	0	0	0	0	0	2	B2	526

[a] The serotype, phylogroup, and ST (MLST sequence type) were determined previously by Balière et al. (47) and Gourmelon et al. (48).

[b] Sampling sites are coded as follows: 1, La Fresnaye, Brittany; 2, Regnéville sur mer, Normandy; 3, La Vanlée, Normandy; 4, Saint-Pol-de-Léon, Brittany; 5, Pirou, Normandy; 6, Sèvre, Poitou-Charente region. Freshwater samples are coded as follows: W1.1, Frémur (lat 48.3711280, long −2.208756); W1.2, Le Rat (lat 48.650571, long −2.1912390); W1.3, Le Clos (lat 48.3728867, long −2.1736788); W1.4, Kermiton (lat 48.3738516, long −2.1716267); W2.1, La Sienne (lat 49.0195333, long −1.5055472); W2.2, La Soulles (lat 49.0234806, long −1.4890194); W2.3, Regnéville sur mer (lat 48.9989694, long −1.5585417); W3.1, La Vanlée (lat 48.9147833, long −1.5498417); W3.2, Les Hardes (lat 48.9309028, long −1.5350306). Seawater samples are coded as W1.5 (Kermiton [lat 48.627192, long −2.291158]). Samples from cockles are coded as C1.1 (Cockles La Fresnaye [lat 48.3750614, long −2.1716026]) or C2.1 (Cockles Regnéville sur mer [lat 48.9716028, long −1.5648194]). Samples from mussels are coded as follows: M1.1, Mussels1 La Fresnaye (lat 48.3825912, long −2.1731444); M1.2, Mussels2 La Fresnaye (lat 48.3812179, long −2.175184); M2.1, Mussels Regnéville sur mer (lat 48.9716444, long −1.5748083); M3.1, Mussels La Vanlée (lat 48.9303583, long −1.5939472); M5.1, Mussels Pirou (lat 49.182884, long −1.6146958); M6.1, Mussels Sèvre (lat 46.29665, long −1.1457804). Samples from oysters are coded as follows: O1.1, Oysters 1 La Fresnaye (lat 48.3820423, long −2.1738512); O1.2, Oysters 2 La Fresnaye (lat 48.388116, long −2.1713438); O3.1, Oysters La Vanlée (lat 48.9303583, long −1.5939472); O4.1, Oysters Saint-Pol-de-Léon (lat 48.674128, long −3.970621). Sediment samples are coded as S3.1 (Sediment La Vanlée [lat 48.9147833, long −1.5498417]).

[c] Genetic marker groups are as follows. The adhesion group includes *eibG*, *iha*, *saa*, *toxB*, *orfA*, *orfB*, *paa*, *stcE*, *sab*, *efa1* or *lifA*, *bfpA*, *espP*, the F6/F987P gene (*fasA*), the F18/F107 gene (*fedA*), the F41 gene (*fimF41a*), $lpfA_{O157}$, $lpfA_{O26}$, $lpfA_{O113}$, *ehaA*, and *epeA*. The type III secretion system group includes *espB*, *espD*, *espA*, *tir*, *espZ*, *escC*, *espG*, *escD*, *escN*, *escV*, *escJ*, *nleB*, *nleE*, *nleF*, *nleG*, *nleH1-2*, *nleA*, *espM1*, *nleC*, *nleD*, *nleH1-1*, *nleG2*, *nleG5-1*, *nleG5-2*, *nleG6-2*, *espV*, *espK*, *espN*, *espJ*, and *espM2*. The toxin group includes *subA*, *ent*, *ehxA*, *cdt-I*, *cdt-III*, *cdt-V*, *sta* (*estIa*), *lt* (*elt*), *hlyA*, *cnf1*, *cnf2*, and *astA*. The "other function" group includes *katP*, *ecf1*, *pagC*, *terE*, *ureD*, *aggR*, *pic*, *irp2*, *fyuA*, Z2098, ECs1763, ECs1822, and *etpD*. The OI-71 pathogenicity island includes *nleF*, *nleG*, *nleH1-2*, *nleA*, and *espM1*; OI-122 includes *efa1* or *lifA*, *nleB*, *nleE*, *ent*, and *pagC*; OI-57 includes *nleG2*, *nleG5-1*, *nleG5-2*, *nleG6-2*, Z2098, and ECs1763; OI-43/48 includes *iha*, *terE*, and *ureD*; the HPI includes *irp2* and *fyuA*. EHEC markers include *espV*, *espK*, *ureD*, and Z2098. The *nle* gene group includes *nleB*, *nleE*, *nleF*, *nleG*, *nleH1-2*, *nleA*, *nleC*, *nleD*, *nleH1-1*, *nleG2*, *nleG5-1*, *nleG5-2*, and *nleG6-2*.

[d] ND, not done.

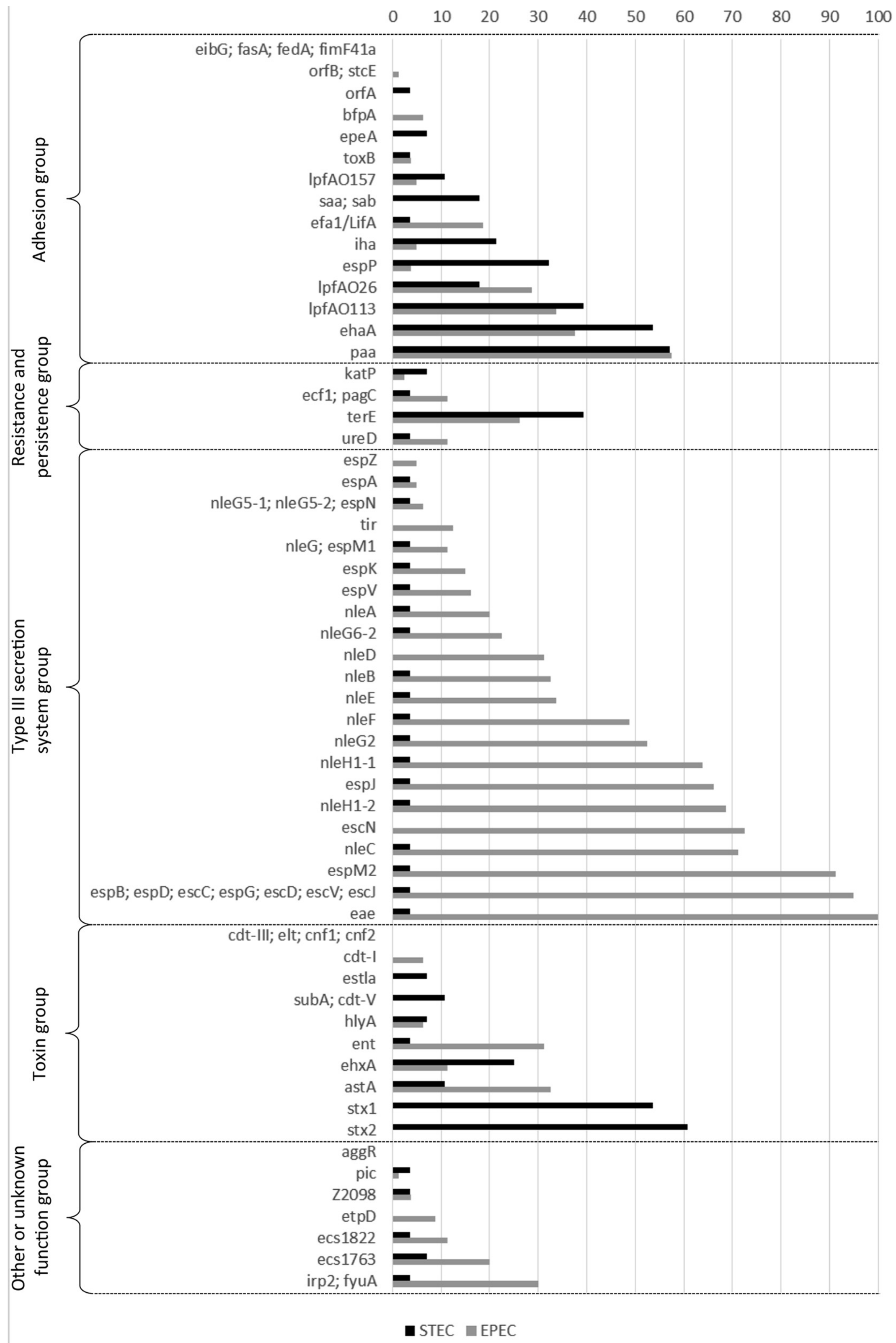

FIG 2 Prevalence of virulence gene targets among STEC and EPEC strains. Each result is expressed as the percentage of strains bearing the indicated gene.

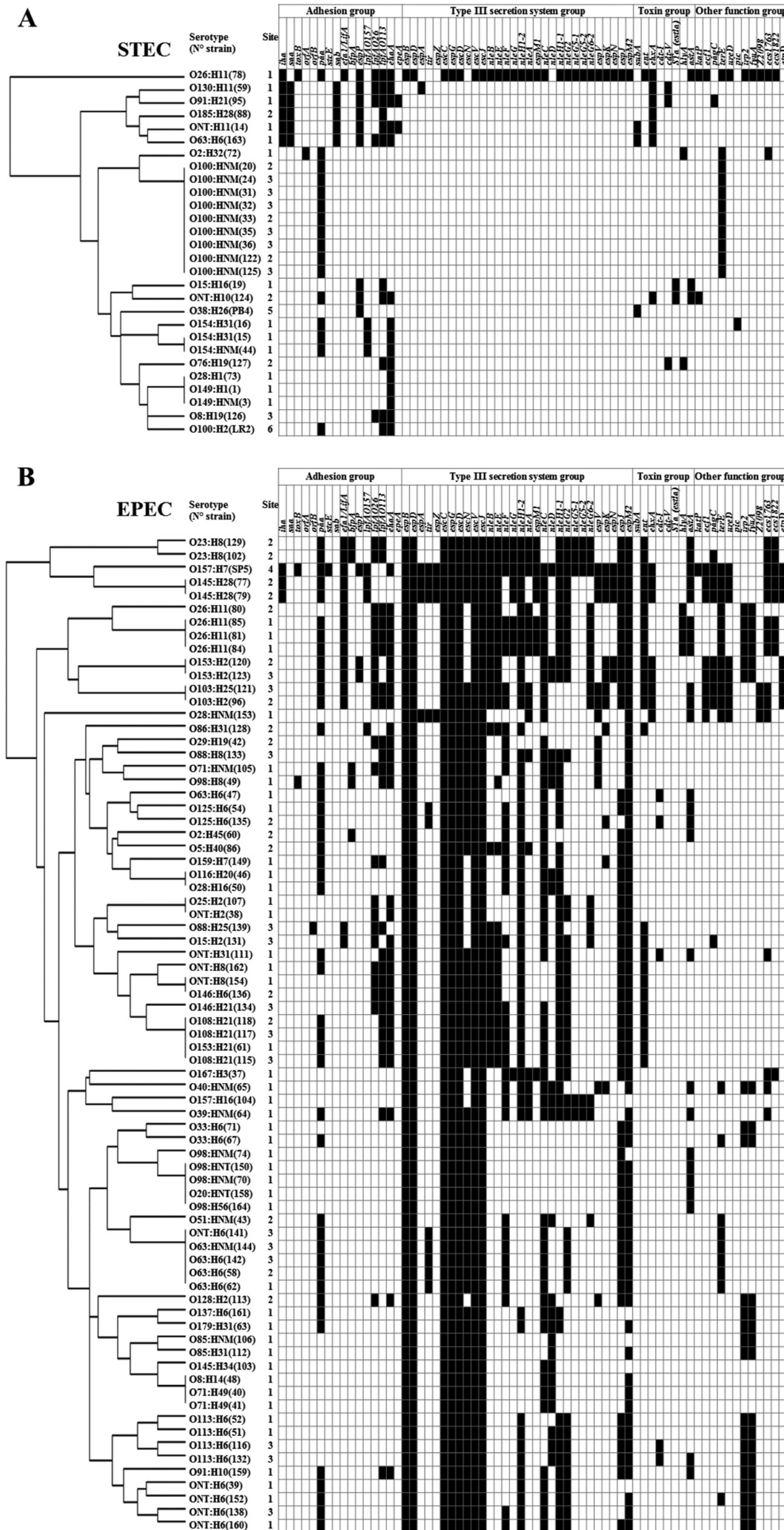

FIG 3 Tree and presence/absence matrix for virulence genes target detected in STEC (A) and EPEC (B) strains. The trees were generated using the heat map hierarchical clustering method with the R software (GitHub). Black or white squares in matrices indicate the presence or absence of a virulence gene target, respectively. The virulence gene targets *eibG*, F6/F987P gene (*fasA*), F18/F107 gene (*fedA*), F41 gene (*fimF41a*), *cdt-III*, *lt* (*elt*), *cnf1*, *cnf2*, and *aggR* are not listed in this figure.

(from a shellfish sample), associated with the stx_{2g} subtype and four supplementary virulence genes (two toxins and two adhesion factors), could potentially be linked with cattle sources, as has been shown previously for similar strains (41, 66). We can hypothesize that this strain is associated with a low human risk. Similarly, the O38:H26 STEC strain isolated from shellfish was associated with a low human risk; indeed it was associated with the stx_{1c} subtype and only one other toxin gene (*subA*) and one adhesion gene (*espP*) and may potentially derive from a sheep source, as shown previously (64, 67). Finally, the stx_{1d} subtype dominates (23% of detected *stx* genes) in STEC strains isolated in our study (from shellfish and freshwater samples). This subtype does not appear to be common in STEC strains (62–64). However, a proportion of stx_{1d} variants similar to those observed here has been observed in STEC strains from ruminant stools in India (18.7%) (68). Little is known of the clinical significance of this subtype, but it seems to be associated with a low risk (69). In agreement with this suspected low virulence, STEC strains with stx_{1d} were found to be associated with only one to four additional virulence genes from the panel of 75 genes investigated.

Among the EPEC strains isolated in this study, some appear potentially pathogenic for humans as a result of the high number and the composition of virulence genes they possess. First, the *E. coli* O157:H7 strain isolated from shellfish possessed the highest number of virulence genes (50 genes), and especially the *ehxA*, *astA*, *lpfA*, *katP*, *etpD*, *espP*, *terE*, and *ureD* genes, identified by using whole-genome sequencing analysis for STEC O157:H7 strains and the EPEC O157:H7 strains isolated from patients with gastrointestinal complaints in the Netherlands (70). These EPEC strains were mostly related to the STEC group and might be referred to as EHEC strains that have lost the Shiga toxin (EHEC-LST [70]). Another category of such strains could be "EHEC-like," as proposed previously for strains of the O26:H11 serotype (71). In addition, strains with non-O157 serotypes (such as O145:H28, O103:H25, O103:H2, and O26:H11) isolated in this study were also found to possess a high number of genes (27 to 39 virulence genes) and could also be regarded as EHEC strains that have lost the Shiga toxin. The large number of virulence genes in these strains is consistent with the fact that members of the top five serotypes are the strains most frequently associated with human diseases (5). Among the strains with the highest number of virulence genes (>27 virulence genes), there are also strains of serotypes O153:H2 and O23:H8. STEC strains of the O153:H2 serotype have already been isolated from human patients (60), whereas EPEC strains that belong to serotype O23:H8 and show similarities to strains in the present study (with the same ST [ST327], genes encoding the same adhesins [$lpfA_{O26}$, $lpfA_{O113}$, and *paa*], and genes of OI-122) were previously associated with nonbloody diarrhea (72).

Among the strains with the highest numbers of virulence genes, several could be potentially pathogenic, since specific virulence gene associations were found. For example, detection of the four genetic markers *espK*, *espV*, *ureD*, and Z2098 in strains belonging to the O103:H2 and O103:H25 serotypes suggests that these strains, isolated from freshwater samples, could be highly virulent for humans, as proposed previously by studies in the detection of EHEC strains and their *stx*-negative derivative strains (30) and in the prediction of strain virulence (29).

Furthermore, 10 strains displayed more than 60% of the genetic markers related to the pathogenicity islands OI-122 (*efa1*, *pagC*, *nleB*, *nleE*, and *ent*), OI-57 (*nleG2*, *nleG5-1*, *nleG5-2*, *nleG6-2*, Z2098, and ECs1763), OI-71 (*espM1*, *nleA*, *nleF*, *nleG*, and *nleH1-2*), and OI-43/48 (*iha*, *terE*, and *ureD*) and to the high-pathogenicity island (HPI) (*irp2* and *fuyA*), which are used to identify strains with the ability to cause severe disease outbreaks (22, 43). These strains were isolated from shellfish and freshwater samples and belonged to serotypes O23:H8, O26:H11, O103:H25, O103:H2, O145:H28, and O157:H7. They are among the strains described above as having the largest number of virulence genes and represent a group of strains with a high virulence potential for humans. This is also corroborated by their serotype, which is associated with the classical EHEC serotype.

The presence of *nle* (non-LEE effector) genes and the number of genes carried by an *E. coli* strain are important criteria for estimating its virulence potential (27, 73). Eight strains carried at least 10 of the 13 *nle* genes analyzed. Most of these strains belong to the O26:H11, O23:H8, and O157:H7 serotypes described above, while two strains of serotypes O157:H16 and O39:HNM were isolated from shellfish and freshwater samples.

The majority of the EPEC strains isolated in this study were found to possess the four main intimin subtypes, which could be highly related to pathogenic serotypes (*eae* subtypes β, γ, ε, and θ, found in 72% of the EPEC strains [17]). The *eae*-γ1 subtype was found in the serotype O157:H7 strain and the two serotype O145:H28 strains, while *eae*-β1 was found in the four strains of serotype O26:H11 (isolated from shellfish and freshwater samples). These correlations are consistent with results obtained previously for strains belonging to the top five EHEC serotypes that were isolated from slaughtered adult cattle (11) and for strains linked to human diseases (51). These data are also consistent with publications that showed that the *eae*-β and *eae*-γ subtypes were the two subtypes most frequently detected in clinical isolates associated with human infections (15, 51, 74).

Some correlations were found between the *eae* subtype and specific virulence genes or phylogroups. All EPEC strains with the *eae*-γ1 subtype, and only those, harbored the *espA* and *espZ* genes. Although from different serotypes, the three *eae*-γ1-containing strains assigned to phylogroups were from phylogroup D and harbored the *tir* gene, which was also detected in seven of the nine *eae*-α2 variants. Similarly, all *eae*-β2 strains ($n = 12$) were from phylogroup B2 and contained the *fyuA* and *irp2* genes (*HPI*), while only 18% of other EPEC variant strains harbored these genes. This resulted in a greater proportion of strains carrying these *fyuA* and *irp2* genes among members of phylogroup B2. Conversely, strains from phylogroup B1 preferentially carried the *ehaA* (89.2%), $lpfA_{O113}$ (83.8%), $lpfA_{O26}$ (70.3%), *nleE* (62.2%), *nleB* (59.5%), and *ent* (59.5%) genes, in contrast to strains of phylogroups A, B1, and D (<15%).

In this study, STEC and EPEC strains were found together in some samples, suggesting that a mixture of STEC and EPEC strains could be present in an environmental sample, thus providing the opportunity for horizontal gene transfer of multiple virulence factors, including gain or loss of *stx* genes. Furthermore, in a selection of water samples, stx_1- or stx_2-converting bacteriophages were searched for by real-time PCR and were found to be present at the same time as STEC or EPEC strains in the samples tested (M. Muniesa, personal communication). These results suggest that new pathogens could emerge as a result of the simultaneous presence and recombination of STEC, EPEC, and *stx*-converting bacteriophages.

In conclusion, this molecular risk assessment study of STEC and EPEC strains isolated from the coastal environment used genetic markers associated with human or nonhuman animal diseases to evaluate the potential risk for shellfish consumers. Seven STEC strains (corresponding to profiles 1 to 5, 7, and 9) were associated with a probable virulence potential for humans either because they displayed a large number of virulence genetic markers or because they harbored stx_{2a}, whether in addition to the stx_{2c} and/or stx_{2d} variant or not. Fifteen EPEC strains (corresponding to profiles 1 to 9 and 36), which could be "EHEC-like," displayed a large number or a particular combination of virulence genetic markers (i.e., >60% of genetic markers related to pathogenicity islands OI-122, OI-57, OI-71, OI-43-48, and HPI or at least 10 of the 13 *nle* genes tested), suggesting an association with human or nonhuman animal infections. This study clearly highlights the ubiquitous presence of potentially pathogenic STEC and EPEC strains in coastal environments (shellfish, water, and sediment samples), even if these strains are less prevalent in such environments than in upstream watersheds as a result of the distance from the source and the negative impact of a saline environment. Risk of a human infection by STEC caused by shellfish consumption seems to be limited because a depuration step or relaying step has to be performed before shellfish from category B and C areas, respectively, reach market (1, 47). To date, no shellfish outbreak involving STEC or EPEC strains has been described. The sanitary classification of shellfish-harvesting areas in Europe is probably an important measure that helps to prevent shellfish food-borne outbreaks caused by these bacteria. However, the absence of a case description could also be linked to an underestimation of the hazard associated with potentially pathogenic STEC and EPEC strains. Therefore, the acquisition of data on circulating strains in the environment is crucial for preventing the risk of human infection and improving our understanding of STEC and EPEC.

ACKNOWLEDGMENTS

We thank Huw Taylor (University of Brighton, Brighton, United Kingdom) for critical review of the manuscript, Maite Muniesa (University of Barcelona, Barcelona, Spain) for providing the results of stx_1- or stx_2-converting bacteriophage detection, and Eliette Riboulet (Université de Caen-Normandie, Caen, France) for help in producing the dendrograms shown in Fig. 3 using the R software.

FUNDING INFORMATION

This work was funded by the European Regional Development Fund Interreg IVA Programme, as part of the collaborative project RiskManche. The thesis of Charlotte Balière was supported by a grant from Institut Français de Recherche pour l'Exploitation de la Mer (Ifremer) and the Agence de l'Eau Loire-Bretagne.

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