AEM
Journals.ASM.org

Prevalences of Shiga Toxin Subtypes and Selected Other Virulence Factors among Shiga-Toxigenic *Escherichia coli* Strains Isolated from Fresh Produce

Peter C. H. Feng,[a] Shanker Reddy[b]

Division of Microbiology, U.S. FDA, College Park, Maryland, USA[a]; Monitoring Programs Division, USDA, Manassas, Virginia, USA[b]

Shiga-toxigenic *Escherichia coli* (STEC) strains were isolated from a variety of fresh produce, but mostly from spinach, with an estimated prevalence rate of 0.5%. A panel of 132 produce STEC strains were characterized for the presence of virulence and putative virulence factor genes and for Shiga toxin subtypes. About 9% of the isolates were found to have the *eae* gene, which encodes the intimin binding protein, and most of these belonged to known pathogenic STEC serotypes, such as O157:H7 and O26:H11, or to serotypes that reportedly have caused human illness. Among the *eae*-negative strains, there were three O113:H21 strains and one O91:H21 strain, which historically have been implicated in illness and therefore may be of concern as well. The *ehxA* gene, which encodes enterohemolysin, was found in ~60% of the isolates, and the *saa* and *subAB* genes, which encode STEC agglutinating adhesin and subtilase cytotoxin, respectively, were found in ~30% of the isolates. However, the precise roles of these three putative virulence factors in STEC pathogenesis have not yet been fully established. The stx_{1a} and stx_{2a} subtypes were present in 22% and 56%, respectively, of the strains overall and were the most common subtypes among produce STEC strains. The stx_{2d} subtype was the second most common subtype (28% overall), followed by stx_{2c} (7.5%), and only 2 to 3% of the produce STEC strains had the stx_{2e} and stx_{2g} subtypes. Almost half of the produce STEC strains had only partial serotypes or were untyped, and most of those that were identified belonged to unremarkable serotypes. Considering the uncertainties of some of these Stx subtypes and putative virulence factors in causing human illness, it is difficult to determine the health risk of many of these produce STEC strains.

Increases in the consumption of fresh produce have resulted in increases in food-borne outbreaks and illness associated with these products, prompting federal agencies to monitor the microbial quality of fresh produce. The FDA has implemented import and domestic compliance programs to check produce samples for the presence of pathogens. Also, the USDA Agricultural Marketing Service initiated the Microbiological Data Program (MDP) in 2001 to conduct microbial surveys of fresh produce samples collected from wholesale distribution centers across the country. On average, 10,000 to 15,000 samples were tested by MDP yearly for the presence of *Salmonella*, enterotoxigenic *Escherichia coli* (ETEC), *E. coli* serotype O157:H7, and other Shiga-toxigenic *E. coli* (STEC) types. The annual MDP reports (http://www.ams.usda.gov/AMSv1.0/mdp) showed that many of these bacteria can be found in various types of fresh produce.

STEC strains are characterized by the production of Shiga toxins (Stx), of which there are two main types, designated Stx1 and Stx2. Within each toxin are many subtypes; currently, there are three known Stx1 (Stx1a, Stx1c, and Stx1d) and seven known Stx2 (Stx2a, Stx2b, Stx2c, Stx2d, Stx2e, Stx2f, and Stx2g) subtypes (1). Some of these subtypes have thus far been found mostly in environmental or animal STEC strains and have not caused human illness (2, 3). There are several hundred known STEC serotypes that can produce any of the Stx subtypes or any combination of subtypes, but not all have been implicated in illness. The production of Stx alone, without an adherence factor, is deemed to be insufficient to cause severe disease. In contrast, enterohemorrhagic *E. coli* (EHEC), a subset of STEC, is composed of pathogenic strains that carry other virulence factors. Most notable of these factors is the production of intimin protein, encoded by the *eae* gene, which enables the pathogen to attach to epithelial cells. The presence of *eae* and stx_2 has been determined to be an important predictor that STEC strains may cause severe disease, such as hemolytic-uremic syndrome (HUS) (4). Serotype O157:H7 is the prototypic EHEC strain, but others, in serogroups O26, O111, and O103, to name a few, have *eae* and have caused severe illness. There are also other EHEC strains, such as strains of serotypes O113:H21 and O91:H21, that do not have *eae* but have caused HUS (5). These *eae*-negative EHEC strains are postulated to have other putative adherence and virulence-associated factors, which include STEC agglutinating adhesin (Saa) and subtilase cytotoxin (SubAB) (5). Many EHEC and STEC strains also produce enterohemolysin, encoded by the plasmid-borne *ehxA* gene, but its role in pathogenesis remains uncertain (4, 6).

The produce-associated STEC strains isolated by the MDP were tested for the presence of stx_1, stx_2, *eae*, and *ehxA* by the *E. coli* Reference Center at Penn State University. The STEC strains isolated by the FDA and the contracting labs were screened mainly for stx_1, stx_2, and serotype O157:H7-specific targets. To better characterize these STEC strains isolated from produce, we tested them for the presence of other STEC virulence and putative virulence factors. Furthermore, studies have examined the prevalences of Stx subtypes among STEC strains isolated from clinical, animal, and some food sources, but not from produce. Hence, we also

Received 23 July 2013 **Accepted** 28 August 2013
Published ahead of print 30 August 2013
Address correspondence to Peter C. H. Feng, peter.feng@fda.hhs.gov.

doi:10.1128/AEM.02455-13

TABLE 1 Distribution and serotypes of some STEC strains isolated from various produce types

Commodity	No. (%) of isolates	Serotype(s)[a]
Cantaloupe	3 (2.2)	Ont:H11, Ont:H52, O88:H38
Chives	1 (0.7)	See Table 3
Cilantro	18 (13.6)	Ont:H16, Ont:H31, Ont:H49, Ont:H52, O1:H+, O8:H16, O8:H28, O113:H5, O139:H1, O153:H21, O168:H8
Coriander	3 (2.2)	Ont:H7, O2:H25, O119:H4
Hot peppers	3 (2.2)	O8:H9, O24:H11, O180:H14
Lettuce	28 (21.2)	Ont:Hnt, O6:H49, O8:H28, Ont:H2, Ont:H8, O136:H16, O143:H34, O163:H19, O168:H−, O168:H8, O181:H49
Parsley	1 (0.7)	Ont:H38
Spinach	70 (52.6)	Ont:H2, Ont:H11, Ont:H16, Ont:H19, Ont:H21, Ont:H28, Ont:H38, Ont:Hnt, O8:H−, O8:H28, O11:H15, O21:Hnt, O76:H+, O88:Hnt, O98:H36, O107:Hnt, O113:H36, O130:H11, O159:H19, O181:H49
Sprouts (alfalfa)	3 (2.2)	Ont:H28, Ont:Hnt, O36:H14
Tomatoes	3 (2.2)	Ont:Hnt
Total	132	

[a] Excludes serotypes listed in Table 2. Also, some serotypes were isolated multiple times from the same type of produce. nt, not typed.

tested these produce STEC strains for the prevalence of specific Stx subtypes.

MATERIALS AND METHODS

Bacterial strains. The 132 produce STEC strains used in this study consisted of 105 strains from the MDP analysis of produce samples from 2002 to 2012, 17 strains from analyses performed by private laboratories under contract by the FDA, and 10 strains from produce testing done by the FDA. The O157:H7 strain from the 2006 spinach outbreak was included in the study for comparative purposes to show the genes or subtypes carried by this strain.

Characterization. All MDP strains and some of the other isolates were serotyped by the *E. coli* Reference Center at Penn State University. Although many of the STEC isolates had already been tested for the stx_1, stx_2, *eae*, and *ehxA* genes, the specificities of the primers used were unknown, so it was uncertain that all the various *eae* alleles and Stx subtypes were detected. Hence, the isolates were retested by a 5P multiplex PCR assay to verify the presence of the stx_1, stx_2, and *ehxA* genes (7). The *stx* primers used in the 5P assay have been shown to detect all *stx* subtypes except for stx_{1d} and stx_{2f} (8), and the *ehxA* primers detect both cluster I and II *ehxA* genes (7).

There are reportedly about 30 *eae* alleles, designated by Greek letters and carried by various STEC strains. To confirm the presence of the *eae* gene among the produce STEC strains, *eae*-specific primers designed from the homologous regions within 15 different *eae* alleles were used for analysis (9). Strains found to have *eae* were further tested with *eae* allele-specific PCR primers (data not shown).

STEC strains that did not have *eae* were tested for the presence of other putative virulence genes. Characterization of *eae*-negative O113:H21 strains identified Saa, encoded by the *saa* gene, as a putative adherence protein (5) but also found SubAB, encoded by the *subAB* gene (10), as a putative virulence factor. Both of these genes are prevalent in *eae*-negative STEC strains, so produce STEC strains that did not have *eae* were tested using *saa*-specific (11) and *subAB*-specific (10) PCR primers.

In light of an outbreak in the European Union caused by Stx-producing enteroaggregative *E. coli* (EAEC) of serotype O104:H4 that implicated sprouts, the panel of produce STEC strains was also tested by PCR for the presence of the *aggR* gene (12), which encodes the transcriptional activator of virulence genes in EAEC strains.

Stx subtyping. STEC strains that were confirmed to carry either stx_1, stx_2, or both were tested by PCR to determine the specific *stx* subtypes. The subtype-specific primers and the PCR protocol used were described by Scheutz et al. (1). Subtypes stx_{2a}, stx_{2c}, and stx_{2d} have been found to be very closely related, and primer cross-reactivity has been reported to occur (1). Thus, strains found to carry 2 or all 3 of these subtypes, especially stx_{2c} and stx_{2d}, were retested with a 66°C annealing temperature instead of 62°C, as prescribed by Scheutz et al. (1).

RESULTS

The various produce commodities from which STEC strains were isolated are summarized in Table 1. About 52% of the STEC strains tested were isolated from spinach, but many were also isolated from lettuce (21%) and cilantro (14%). Of the 132 STEC strains, 56 yielded partial serotype data, where either the O or H antigen or both could not be identified. Some of the STEC serotypes observed are shown in Table 1, and those that are known pathogenic serotypes or serotypes that have historically been isolated from infections (13) are listed in Table 2. More details on the MDP STEC strains can be found in the MDP reports (http://www.ams.usda.gov/AMSv1.0/mdp).

Results of our PCR analyses for *stx*, *eae*, and *ehxA* were mostly consistent with reported data, but there were some discrepancies. A few strains reported to be STEC were found to not carry *stx* genes, and a few strains reported to have stx_1 actually had stx_2 and vice versa (data not shown). A similar discrepancy was observed for the presence or absence of *ehxA* in some strains. In total, 81/132 (61%) produce STEC isolates examined had the *ehxA* gene. All strains reported to have the *eae* gene were confirmed by our assay, and in addition, two strains not reported to have *eae* were found to carry the *eae* gene. Excluding the spinach *E. coli* O157:H7 strain, 12/132 (9.0%) produce STEC strains had *eae* (Table 2), and these consisted of 5 O157:H7 strains that had γ-*eae*, two *E. coli* O26:H11 strains with β-*eae*, two O165:H25 strains and an O121:H19 strain with ε-*eae*, and two other strains whose *eae* alleles could not be typed (Table 2). Analysis for the other putative virulence factors in *eae*-negative strains showed that 35% (43/120 strains) had the *saa* gene and 32% (38/120 strains) had the *subAB* gene (Table 3). STEC strains often had both of these genes, except for a few strains that had one or the other. Lastly, none of the 132 produce STEC strains were found to be positive for the *aggR* gene (Table 3).

Analysis for Stx subtypes showed that 16/132 (10%) produce STEC strains had stx_1 only (Table 3), and subtyping PCR showed that of those, 14/16 (87%) had the stx_{1a} subtype. The stx_{1a} subtype was also predominant among strains that had multiple subtypes,

TABLE 2 Serotypes and pathotypes of selected STEC strains from produce[a]

Commodity	Serotype	Pathotype
Cherry tomato	O8:H19[b]	stx_{1a} stx_{1c} stx_{2a} *ehxA subA/B*
Chives	**O157:H7**	stx_{1a} stx_{2a} γ-*eae ehxA*
Cilantro	O20:H19[b]	stx_{1a} stx_{2d} *saa ehxA*
	O26:H11	stx_{1a} β-*eae ehxA*
	O165:H25	stx_{1a} stx_{2a} *ehxA* ε-*eae*
	Untyped	stx_{1a} *eae*[g] *ehxA*
Lettuce	O2:H27[c]	stx_{2a} *ehxA*
	O121:H19	stx_{2a} *ehxA* ε-*eae*
	O157:H7	stx_{2a} γ-*eae*
	O157:H7	stx_{2a} γ-*eae ehxA*
	O163:H19[b]	stx_{2a} stx_{2d} *ehxA subA/B*
	O165:H25	stx_{1a} stx_{2a} *ehxA* ε-*eae*
	O174:H21[b]	stx_{2a}
Spinach	**O157:H7**[f]	stx_{2a} stx_{2c} γ-*eae ehxA*
	O8:H19[b]	stx_{2a} stx_{2d} *ehxA*
	O8:H19[b]	stx_{2a} *ehxA*
	O26:H11	stx_{1a} stx_{1c} β-*eae ehxA*
	O82:H8[c]	stx_{2a} *ehxA saa*
	O82:H8[c]	stx_{2a} *ehxA saa*
	O91:H21	stx_{2a} *ehxA saa*
	O98:H36[d]	stx_{1a} *eae*[h] *ehxA*
	O113:H21	stx_{2a} *ehxA saa subAB*
	O113:H21	stx_{2a} stx_{2d} *ehxA saa subAB*
	O113:H21	stx_{2a} stx_{2d} *ehxA saa subAB*
	O116:H21[e]	stx_{2a} *ehxA saa subAB*
	O157:H7	stx_{2a} γ-*eae ehxA*
	O157:H7	stx_{2a} stx_{2c} γ-*eae ehxA*
	O174:H2[b]	stx_{2a} *ehxA saa*
	O174:H21[b]	stx_{1a} stx_{2a} stx_{2d} *ehxA saa subAB*

[a] The data listed include known pathogenic serotypes (in bold) and serotypes that have reportedly been isolated from patients with HUS and other illness.
[b] Serotype reportedly isolated from patients with HUS (13).
[c] Serotype reportedly isolated from patients with other illnesses (13).
[d] Serotype has no history of causing illness (13).
[e] Serotype reportedly isolated from patients with HUS (10).
[f] Cause of spinach outbreak of 2006. This strain was included for reference.
[g] *eae* allele is undetermined.
[h] *eae* allele is undetermined but is not the α, β, ε, or γ allele.

TABLE 3 Prevalence of *stx* subtypes and virulence and putative virulence genes among produce STEC strains

Gene[a]	Subtype(s)	No. (%) of isolates (n = 132)[b]
stx	1a	14 (10.5)
	1c	2 (1.5)
	1d	ND
	2a	46 (34.6)
	2b	ND
	2c	5 (3.7)
	2d	15 (11.7)
	2e	4 (3.0)
	2f	ND
	2g	3 (2.2)
	1a, 1c	2 (1.5)
	1a, 2a	5 (3.7)
	1a, 2c	2 (1.5)
	1a, 2d	3 (2.2)
	1c, 2b	1 (0.7)
	2a, 2d	17 (12.8)
	2a, 2c	3 (2.2)
	1a, 1c, 2a	1 (0.7)
	1a, 2a, 2d	2 (1.5)
eae		12 (9.0)
ehxA		81 (60.9)
saa		43 (35)[c]
subAB		38 (32)[c]
aggR		0 (0.0)

[a] *stx*, Shiga toxin gene; *eae*, intimin gene; *ehxA*, enterohemolysin gene; *saa*, STEC agglutinating adhesin gene; *subAB*, subtilase cytotoxin gene; *aggR*, EAEC virulence regulator gene.
[b] ND, not detected.
[c] Percentage was calculated based on number of *eae*-negative strains (n = 120).

and except for 1 strain that had stx_{1c} and stx_{2b}, the stx_{1a} subtype was found in 15/16 strains that had combinations of subtypes (Table 3). The other stx_1 subtypes were rare among the produce STEC strains, with only 2 strains (1.5%) that had stx_{1c} alone and 2 others that had stx_{1a} and stx_{1c}. None of the STEC strains had the stx_{1d} subtype.

The stx_{2a} subtype was the most common and was found in 35% (46/132 strains) of the produce STEC strains overall (Table 3); among the strains with Stx2 only, 63% (46/73 strains) had stx_{2a}. The overall prevalence rates for the other stx_2 subtypes were as follows: stx_{2d}, 11%; stx_{2c}, 3.7%; stx_{2e}, 3%; and stx_{2g}, 2.2% (Table 3). No strains carried stx_{2b} and stx_{2f} alone, but 1 strain had stx_{2b} and stx_{1c}. Among the strains that had multiple stx_2 subtypes, strains with stx_{2a} and stx_{2d} were the most common (17 strains). There were 3 strains that had stx_{2a} and stx_{2c}, and 2 of these were O157:H7 strains. A few other strains had combinations of various stx_1 and/or stx_2 subtypes (Table 3). The specific *stx* subtypes and the virulence and/or putative virulence factors carried by the produce STEC strains that belonged to known pathogenic serotypes and serotypes that historically have been associated with human illness (13) are shown in Table 2.

DISCUSSION

STEC can be found in various environmental and food sources (3, 14–16), and produce is no exception, as many STEC strains have been isolated from produce, especially from spinach, lettuce, and cilantro. To estimate the prevalence rates of STEC in these products, we took the number of STEC strains isolated annually by the MDP in relation to the ~2,200 samples of each commodity tested yearly and determined that STEC was present in 0.5 to 0.6% of the spinach, 0.3 to 0.5% of the cilantro, and 0.04 to 0.18% of the lettuce samples. It should be pointed out, however, that these estimations were based on the MDP data from the last few years and thus may not be indicative of the overall trend. For example, based on data from recent years, the estimated prevalence rate of STEC for lettuce seems low: there were higher isolation rates from lettuce in other years, and considering that 28/132 STEC strains we examined were from lettuce, the 0.04 to 0.18% prevalence rates obtained for lettuce may be underestimated. These observations also suggest that prevalences can vary greatly and depend on many factors, including seasonal and regional variations.

The estimated prevalence rate of 0.5% for spinach, however, seems fairly stable, as 11 to 14 STEC strains were isolated each year from 2009 to 2011 and 21 STEC isolates (0.95%) came from spinach in 2012. Spinach testing was initiated by the MDP in 2008 in

response to the O157:H7 outbreak from spinach in 2006. Yet in only 4 years of testing (2008 to 2012), spinach accounted for over half (56/105 strains) of all STEC isolations. While many of these STEC serotypes were unremarkable, with no history of having caused human illness, their prevalence suggests that there may be some correlation between STEC and spinach plants or spinach cultivation and processing practices.

The *ehxA* gene, which encodes enterohemolysin, was detected in 61% of the produce STEC strains. This is consistent with reports that this gene is common among STEC strains. Analysis of 343 STEC strains isolated from clinical patients in Denmark showed that 77% had the *ehxA* gene, but its presence could not be correlated with the occurrence of HUS or bloody diarrhea (4). The *ehxA* gene is also prevalent among STEC strains in the environment, as it was found in 30 to 63% of STEC isolates from farm animals (6, 15), and ~40% of STEC strains from deer carried *ehxA* (2, 17). Analysis of STEC isolates from foods showed that 33% of the strains isolated from Swiss raw milk cheeses (18) and 40% of those isolated from ground beef, sausages, and milk in Germany (19) had *ehxA*. A recent U.S. study of 338 STEC isolates from commercial ground beef showed an even higher prevalence rate, as 78% of the STEC strains were found to have *ehxA* (20). Hence, our finding that ~60% of the produce STEC strains had *ehxA* is consistent with these reports. Almost all the known and reportedly pathogenic STEC strains from produce had *ehxA* (Table 2), but there were a few exceptions. Serotype O157:H7 strains almost always had the *ehxA* gene, but an O157:H7 strain isolated from lettuce did not (Table 2). Thus far, the role of *ehxA* in STEC pathogenesis is uncertain, as the virulent sorbitol-fermenting O157 strains that caused many HUS outbreaks in the European Union did not express *ehxA* (21). Furthermore, an analysis of ~300 generic *E. coli* strains isolated from water from various states in the United States showed that all had and expressed *ehxA* and none had *stx* genes (22). Except for the absence of *ehxA*, the lettuce isolate was a typical O157:H7 strain that had stx_2 and γ-*eae*, so it was most likely pathogenic.

The *saa* and *subAB* genes are putative virulence factors that are found mostly in *eae*-negative STEC strains (10, 23, 24). Consistently, neither gene was present in produce STEC strains that had *eae*, and among the *eae*-negative strains, 35% and 32% had the *saa* and *subAB* genes, respectively. The plasmid-borne *saa* gene was first identified in an *eae*-negative O113:H21 strain that was implicated in an outbreak of HUS in Australia (5). The Saa protein was determined to be an adherence factor, as a plasmid-cured, *saa*-negative O113:H21 mutant showed reduced adherence compared to the wild type, and the purified Saa protein enhanced adherence to HEp-2 cells (25). Jenkins et al. (26) examined the distribution of the *saa* genes and determined that although *saa* was found in some clinical STEC strains, there was no significant correlation between the presence of *saa* and HUS, and moreover, many STEC strains isolated from healthy cattle had *saa*. These findings were supported by others, which showed that 40% and 53% of the *eae*-negative STEC strains from humans and cattle, respectively, carried *saa* (27). Similarly, another study showed that ~30% of the STEC strains isolated from cattle and meats had *saa* (24, 28), but among *eae*-negative strains, as many as 48% carried the gene (24). Our finding that 35% of produce STEC strains have *saa* is consistent with the fact that this gene is common among STEC strains but provides no further insight into the role of this adherence factor in pathogenesis.

The subtilase cytotoxin encoded by the *subAB* gene has been determined to be a potent toxin that is even more cytotoxic to Vero cells than Stx is (29). It is also found predominantly in *eae*-negative STEC strains, but its role in the pathogenesis of *eae*-negative STEC strains also remains uncertain. For instance, strains of the O91:H21 and O22:H8 serotypes have caused severe illness, but ground beef STEC isolates of these serotypes did not have *subAB* (20). Consistent with this report, the O91:H21 strain that was isolated from spinach had *saa* but not *subAB* (Table 2). The prevalence of the *subAB* gene among STEC strains varies greatly. A study of environmental STEC strains in Brazil showed that 25% of the ~1,200 strains tested had *subAB*, and the prevalence rates ranged from 3% in isolates from goats to 44% in those from dairy cattle (23). Another study from Brazil tested 121 STEC strains isolated from clinical and animal samples and found *subAB* in 48% of the strains but not in the 49 clinical isolates (30). It should be mentioned, however, that 46/49 clinical isolates examined in that study had *eae* and therefore would not have been expected to have *subAB*. A survey of ~200 *eae*-negative STEC strains in Argentina showed that 36% of the cattle and 32% of the human STEC isolates had *subAB* (27). Even higher prevalence rates were reported by others, as 72% and 86% of the *eae*-negative STEC strains from diarrhea patients and healthy sheep, respectively, were found to carry *subAB* (31). A study of STEC strains from foods showed that 49% of the STEC strains isolated from ground beef in the United States carried *subAB* (20). Thus, our finding that 32% of the produce STEC isolates had the *subAB* gene was not unusual. Both the *saa* and *subAB* genes were originally identified in an O113:H21 strain (11) and have been reported to be prevalent in this serotype (10, 27). There were three O113:H21 strains that were isolated from spinach, and all three carried both *saa* and *subAB* (32). Studies of STEC isolated from animals and ground meats showed that O116:H21 strains often also carried *subAB* (20, 23). There were three O116:H21 strains, all isolated from spinach, and two carried *subAB*, so this gene does appear to be fairly prevalent in this serotype as well. It is uncertain if these O116:H21 strains from spinach were pathogenic, but a strain of the O116:H21 serotype was reported to have been isolated from an HUS patient in Australia (10), suggesting that some strains in this serotype may be pathogenic.

Excluding the O157:H7 spinach outbreak strain included in Table 2 as a reference, the *eae* gene was found in 12 (9.0%) produce STEC strains, and most of these were of known pathogenic serotypes or serotypes that reportedly have caused human illness (13), so it is easy to surmise that these strains are health risks. However, there were a few exceptions, as an O98:H36 strain from spinach and an untyped strain from cilantro both had stx_{1a} and an untyped *eae* allele. Although STEC strains of serotype O26:H11 that have stx_{1a} and β-*eae* have caused infections and are known pathogens, O98:H36 strains have had no history of causing illness. Still, the presence of the *eae* adherence gene and stx_{1a} in the same strain is suggestive that both strains may have the potential to cause disease.

Although *eae* is the predominant adherence factor among EHEC strains, other pathogenic *E. coli* strains utilize different adherence factors. For example, the O104:H4 strain that caused the large HUS outbreak in the European Union was an EAEC strain that was postulated to have acquired the ability to produce Stx. The virulence mechanism of EAEC strains is mediated by the *aggR*-encoded transcriptional activator to enable aggregative at-

tachment of EAEC strains to epithelial cells (12). Analysis of the produce STEC strains showed that none of the strains had *aggR* and therefore were not EAEC strains. Analogous to the situation with *eae*, the presence of *stx* and *aggR* within the same strain would have been of health concern.

The prevalences of Stx subtypes in STEC strains isolated from clinical, animal, and environmental sources have been reported (3, 14, 16, 20, 33), but not those for STEC strains isolated from produce. Before discussing our Stx subtyping results, however, it should be mentioned that some Stx2 subtypes, especially stx_{2a}, stx_{2c}, and stx_{2d}, share DNA sequence homologies, and prior to the proposal of Scheutz et al. (1) in 2012, few attempts were made to standardize Stx nomenclature or subtyping methods. For example, 11 STEC strains isolated from cattle and sheep were originally thought to carry stx_{2c}, but further testing showed that 7 of them actually had the stx_{2d}-activatable subtype (34). Due to the lack of consensus, the subtype designations used in some of the cited studies may be different, which may account for some differences in the prevalence rates of these 3 subtypes.

Of the 132 produce STEC strains examined, 16 strains had stx_1 alone. Of these, none had stx_{1d}; only 2 strains, both Ont:H52 ("nt" = not typed) serotype strains, had stx_{1c} only; and a few others had stx_{1c} in combination with other subtypes. The two Ont:H52 strains were previously characterized and shown to express Stx1, carry the stable toxin gene of ETEC, and belong to a unique clonal group (35). The stx_{1c} subtype does not appear to be common, as a study of STEC isolates from ground beef in the United States showed that only 3/338 strains had stx_{1c} (20). In contrast, however, an analysis of 140 STEC strains isolated from animals, meats, and humans in India showed that the stx_{1c} and stx_{1d} subtypes were present in 10% and 13% of the strains, respectively, and 80% of these strains were isolated from nonclinical sources (33). Little is known about the clinical significance of the stx_{1d} subtype, but stx_{1c} is the most common subtype among STEC strains isolated from sheep and wild deer, and also from wildlife meats (2, 36, 37). The stx_{1c} subtype is usually found in *eae*-negative STEC strains and causes infections that tend to be asymptomatic or manifest as mild diarrhea (38). The stx_{1c} subtype has not yet been detected in O157:H7 strains.

Most produce STEC strains that had Stx1 alone had the stx_{1a} subtype, and this was also the most common subtype found in combination with other, mostly stx_2 subtypes, for an overall prevalence rate of 22% (29/132 strains). Pathogenic STEC strains that produce Stx1 most often have the stx_{1a} subtype, and accordingly, all the produce STEC strains from the known pathogenic serotypes had stx_{1a}. However, except for the O98:H36 strain and the untyped strain already mentioned, all the other produce STEC strains that had stx_{1a} did not have *eae*, but a few had *saa* and/or *subAB*. Considering the uncertainties of these two factors in STEC pathogenesis and the absence of a known adherence factor in these strains, it is difficult to assess if these stx_{1a}-bearing produce STEC strains can cause severe disease.

There were 73 produce STEC strains that had stx_2 alone, and of these, 3 had stx_{2g}, 4 had stx_{2e}, and none had stx_{2b} and stx_{2f}. Also, except for 1 strain that had stx_{1c} and stx_{2b}, these four stx_2 subtypes were not found in combination with other subtypes in any produce STEC strain. The stx_{2f} subtype was originally found in STEC strains from pigeons (39), and genetically, it is very distinct from the other subtypes. The stx_{2f} subtype has not been implicated in disease (2, 40, 41), and it is also rare, as various analyses of STEC strains isolated from the wild, from bovine farm environments, and from humans have not detected this subtype (2, 15, 42). The stx_{2b} subtype was originally proposed to designate a variant of stx_{2c} that was found in STEC strains that did not cause HUS (40), so it appears to not cause severe illness (2). Although stx_{2b} was present in only 1 produce STEC strain and in combination with stx_{1c}, this subtype may be common elsewhere. An analysis of STEC strains isolated from deer droppings and wild deer populations in Switzerland showed that 24/52 isolates examined had stx_{2b} (2). The stx_{2g} subtype was found in only 3 (2%) produce STEC strains, and these were isolated from sprouts, spinach, and coriander. The stx_{2g} subtype was first isolated from bacteriophages in feces-contaminated water (43), and except for a report of a STEC strain with stx_{2g} being isolated from a patient without diarrhea (F. Scheutz, personal communication), it has rarely been found in human strains (36). However, stx_{2g} may not be rare, as it was present in 8.4% (9/107 strains) of the STEC strains isolated from farm environments (15) and also detected in some STEC strains isolated from foods (36).

The stx_{2e} subtype was detected in 4 produce STEC strains isolated from pepper, cilantro, and basil. STEC strains that have stx_{2e} are most often isolated from pigs and pork meats (36) and are commonly associated with pig edema disease (44). Some human STEC strains have occasionally been found to have stx_{2e}, but the prevalence rate is low, and it also exists with similar frequencies between isolates from asymptomatic and diarrheic patients (42). Studies also showed that high production of Stx2e by human STEC isolates had no correlation to diarrheal diseases, suggesting that this subtype is not pathogenic for humans (44). Some of the produce STEC strains that carried stx_{2g} and stx_{2e} also had *ehxA*, but none had *eae*, *saa*, or *subA/B*. Considering the uncertain implications of these Stx subtypes in human disease, these produce STEC strains are most likely of low health risk.

The Stx2 toxin is regarded as more potent than Stx1, and of the Stx2 subtypes, Stx2a, Stx2c, and Stx2d have most often been implicated in severe illnesses such as HUS (40, 45). Among the produce STEC strains examined, the stx_{2a} subtype was the most common and was found in 56% (74/132 strains) of the STEC strains overall; among strains that had stx_2 alone, it accounted for 63% (46/73 strains) of the isolates. Pathogenic STEC strains often produce Stx2a, and accordingly, almost every produce STEC strain that had stx_2 and belonged to a known pathogenic serotype or a serotype that has historically been reported to cause illness had the stx_{2a} subtype (Table 2). Several studies have shown that the stx_{2a} subtype is very prevalent among environmental STEC strains. In Spain, the prevalences of stx_{2a} ranged from 18% in STEC strains isolated from sewage and wastewater sources (14) to 50% in STEC strains isolated from wildlife (16). One study from India showed that over 70% of STEC strains isolated from animal stools had the stx_{2a} subtype (33). Among STEC strains isolated from foods, the stx_{2a} subtype was found in 40% (11/27 strains) of the strains isolated from Swiss raw milk cheeses (18). In the United States, it was found in 26% (89/338 strains) of the STEC strains isolated from ground beef that had Stx2 alone, and in combination with other *stx* subtypes, it was present in 130 other strains, for an overall prevalence rate of 64% (219/338 strains) (20). Our finding that 56% of the produce STEC strains overall had stx_{2a} is consistent with the observation that this subtype is very prevalent among STEC strains isolated from various sources, including produce.

The stx_{2c} subtype has been implicated in human illness, and

stx_{2c}-bearing STEC strains have been associated with severe illnesses such as HUS (40, 42). For example, the O157:H7 strain that caused the large spinach outbreak in the United States in 2006 had both stx_{2a} and stx_{2c}. The prevalence of stx_{2c} varies greatly. A study in India showed that stx_{2c} was the most common subtype among STEC strains isolated from animal stools and was found in 37% of the isolates. A study from Spain showed similar results, where 38% of the STEC strains isolated from cattle fecal wastes had the stx_{2c} subtype (14). An analysis of STEC isolates from ground beef in the United States showed that 11% of them carried stx_{2c} alone, but another 16.5% of the isolates had both stx_{2a} and stx_{2c} (20). Among the produce STEC strains, only 5 isolates (3.7%) had stx_{2c} alone, another 3 strains (2.2%) had stx_{2a} and stx_{2c} (2 of these were *E. coli* O157:H7 strains), and 2 other strains had stx_{1a} and stx_{2c}, so the stx_{2c} subtype was found in only 7.5% of the strains overall and was not very prevalent among this panel of produce STEC strains.

The Stx2d subtype used to be differentiated into the Stx2d and Stx2d-activatable subtypes to distinguish those that can be activated by elastase in the mucus, which increases Vero cell cytotoxicity by several hundredfold (46). The Stx2d-activatable toxin is usually the sole Stx produced by *eae*-negative STEC strains, and it was found to be associated with severe diseases such as bloody diarrhea and HUS (45). The Stx2d-activatable subtype used to be identified by PCR followed by PstI restriction analysis, but the recently proposed sequence-based nomenclature system has grouped the Stx2d-activatable subtype into Stx2d, and some of the Stx2d strains that were nonactivatable have now been reclassified as Stx2b strains (1). The proposed Stx2d nomenclature only implies activation potential; hence, the term "activatable" is still used by some to designate strains that have been shown to be activated by elastase. The stx_{2d} subtype was the second most common stx_2 subtype among produce STEC strains and was found by itself in 11% (15/132 strains) of the strains, but it was also present in another 22 strains that also had other subtypes, mostly stx_{2a}. Thus, stx_{2d} was present in 28% (37/132 strains) of the produce STEC strains overall. The prevalence rate of the stx_{2d} subtype among STEC strains varies greatly, ranging from 0.8% in STEC strains isolated from ground beef in the United States (20) to 9% in strains isolated from human and animal stools in India (33). A Swiss study which used the same *stx* subtyping primers and protocol as this study showed that 9.6% of the STEC strains isolated from wild deer populations had the stx_{2d} subtype (2). Thus, our finding that 11% of the produce STEC strains carried the stx_{2d} subtype is fairly consistent with that report. STEC strains of serotypes O113:H21 and O91:H21 that carry stx_{2d} alone have caused HUS (5, 10). There were 3 spinach isolates of the O113:H21 serotype, but 2 had stx_{2a} and stx_{2d}, and the other had stx_{2a} alone. Similarly, the *E. coli* O91:H21 strain from spinach had only stx_{2a}. Thus, none of these strains had stx_{2d} alone, so it is uncertain if these produce strains are analogous to the stx_{2d}-bearing strains of these serotypes that caused severe illness. Most of the other produce STEC strains that had the stx_{2d} subtype alone were not serotyped or yielded only partial serotypes, and of those that were typed, most belonged to unremarkable serotypes such as O168:H8 and O181:H49, to name a couple. Also, some had the *saa* and *subAB* genes, but there were also many that had neither of these genes nor *ehxA*, and hence, the health risks of these strains are uncertain.

In summary, over half of the produce STEC strains examined were isolated from spinach, and about 9% of this panel of strains had the *eae* gene. These included strains of the O157:H7, O121:H19, O26:H11, and O165:H25 serotypes, which are known pathogens, but also included a few *eae*-bearing strains that had no historical implication in illness. Although most of the produce STEC strains did not have *eae*, there were a few strains of serotypes O91:H21 and O113:H21, which historically have caused severe illness. Over half of the produce STEC strains had *ehxA*, and about a third also had *saa*, *subAB*, or both, but the roles of these genes in STEC pathogenesis remain uncertain. The produce STEC strains carried many different Stx subtypes, with stx_{1a} and stx_{2a} being the most common and being observed either by themselves or in combination with other subtypes. Most of the produce strains that belonged to recognized pathogenic STEC serotypes had the stx_{1a} and stx_{2a} subtypes, and many of these also had *eae*. The stx_{2d} subtype was the second most prevalent subtype among produce STEC strains and was often found by itself or in combination with stx_{2a}. Many of these stx_{2d}-bearing strains were only partially serotyped, were untyped, or belonged to unremarkable serotypes. The stx_{2c}, stx_{2e}, and stx_{2g} subtypes were detected in a few strains, and the other stx_1 and stx_2 subtypes were not detected or were present in only a few produce STEC strains. Considering the uncertainties of some of these Stx subtypes and putative virulence factors in STEC pathogenesis, it is uncertain if many of the *eae*-negative produce STEC strains can cause severe human illness.

ACKNOWLEDGMENTS

We are thankful to the scientists in the many state agriculture labs that participated in the MDP and performed the analysis of produce samples. Their work not only yielded a wealth of valuable data on the presence of pathogens in produce but also produced the large and useful collection of produce STEC strains employed in this study. We also thank L. Teel for helpful discussions on Stx nomenclature.

REFERENCES

1. **Scheutz F, Teel LD, Beutin L, Piérard D, Buvens G, Karch H, Mellmann A, Caprioli A, Tozzoli R, Morabito S, Strockbine NA, Melton-Celsa AR, Sanchez M, Persson S, O'Brien AD.** 2012. Multicenter evaluation of a sequence-based protocol for subtyping Shiga toxins and standardizing Stx nomenclature. J. Clin. Microbiol. **50:**2951–2963.
2. **Hofer E, Cernela NN, Stephan R.** 2012. Shiga toxin subtypes associated with Shiga toxin-producing *Escherichia coli* strains isolated from red deer, roe deer, chamois and ibex. Foodborne Pathog. Dis. **9:**792–795.
3. **Martin A, Beutin L.** 2011. Characteristics of Shiga toxin-producing *Escherichia coli* from meat and milk products of different origins and association with food producing animals as main contamination sources. Int. J. Food Microbiol. **146:**99–104.
4. **Ethelberg S, Olsen KE, Scheutz F, Jensen C, Schiellerup P, Enberg J, Petersen AM, Olesen B, Gerner-Smidt P, Mølbak K.** 2004. Virulence factors for hemolytic uremic syndrome, Denmark. Emerg. Infect. Dis. **10:**842–847.
5. **Paton AW, Woodrow MC, Doyle RM, Lanser JA, Paton JC.** 1999. Molecular characterization of a Shiga toxigenic *Escherichia coli* O113:H21 strain lacking *eae* responsible for a cluster of cases of hemolytic-uremic syndrome. J. Clin. Microbiol. **37:**3357–3361.
6. **Cookson AL, Bennett J, Thomson-Carter F, Attwood GT.** 2007. Molecular subtyping and genetic analysis of the enterohemolysin gene (*ehxA*) from Shiga toxin-producing *Escherichia coli* and atypical enteropathogenic *E. coli*. Appl. Environ. Microbiol. **73:**6360–6369.
7. **Feng P, Monday SR.** 2000. Multiplex PCR for the detection of trait and virulence factors in enterohemorrhagic *Escherichia coli* serotypes. Mol. Cell. Probes **14:**333–337.
8. **Feng PCH, Jinnemann K, Scheutz F, Monday S.** 2011. Specificity of PCR and serological assays in detecting *Escherichia coli* Shiga toxin alleles. Appl. Environ. Microbiol. **77:**6699–6702.
9. **Monday SR, Beisaw A, Feng PCH.** 2007. Identification of Shiga toxigenic *E. coli* seropathotypes A and B by multiplex PCR. Mol. Cell. Probes **21:**308–311.

10. **Newton HJ, Sloan J, Bulach DM, Seemann T, Allison CC, Tauschek M, Robins-Browne RM, Paton JC, Whittam TS, Paton AW, Hartland EL.** 2009. Shiga toxin-producing *Escherichia coli* strains negative for locus of enterocyte effacement. Emerg. Infect. Dis. **15:**372–380.
11. **Paton AW, Paton JC.** 2002. Direct detection and characterization of Shiga-toxigenic *Escherichia coli* strains by multiplex PCR for stx_1, stx_2, *eae*, *ehxA*, and *saa*. J. Clin. Microbiol. **40:**271–274.
12. **Cerna JF, Nataro JP, Estrada-Garcia T.** 2003. Multiplex PCR for detection of three plasmid-borne genes of enteroaggregative *Escherichia coli* strains. J. Clin. Microbiol. **41:**2138–2140.
13. **Hussein HS.** 2007. Prevalence and pathogenicity of Shiga toxin-producing *Escherichia coli* in beef cattle and their products. J. Anim. Sci. **85**(Suppl)**:**E63–E72.
14. **García-Aljaro C, Muniesa M, Blanco JE, Blanco M, Blanco J, Jofre J, Blanch AR.** 2005. Characterization of Shiga toxin-producing *Escherichia coli* isolated from aquatic environments. FEMS Microbiol. Lett. **246:**55–65.
15. **Monaghan A, Byrne B, Fanning S, Sweeney T, McDowell D, Bolton DJ.** 2011. Serotypes and virulence profiles of non-O157 Shiga toxin-producing *Escherichia coli* isolates from bovine farms. Appl. Environ. Microbiol. **77:**8662–8668.
16. **Mora A, López C, Dhabi G, López-Beceiro AM, Fidalgo LE, Díaz EA, Martínez-Carrasco C, Mamani R, Herrera A, Blanco JE, Blanco MM, Blanco J.** 2012. Seropathotypes, phylogroups, Stx subtypes and intimin types of wildlife-carried, Shiga toxin-producing *Escherichia coli* strains with the same characteristics as human-pathogenic isolates. Appl. Environ. Microbiol. **78:**2578–2585.
17. **Díaz-Sánchez S, Sánchez S, Sánchez M, Herrera-León S, Hanning I, Vidal D.** 2012. Detection and characterization of Shiga toxin-producing *Escherichia coli* in game meat and ready-to-eat meat product. Int. J. Food Microbiol. **160:**179–182.
18. **Zweifel C, Giezendanner N, Corti S, Krause G, Beutin L, Danuser J, Stephan R.** 2010. Characteristics of Shiga toxin-producing *Escherichia coli* isolated from Swiss raw milk cheese within a 3-year monitoring program. J. Food Prot. **73:**88–91.
19. **Slanec T, Fruth A, Creuzburg K, Schmidt H.** 2009. Molecular analysis of virulence profiles and Shiga toxin genes in food-borne Shiga toxin-producing *Escherichia coli.* Appl. Environ. Microbiol. **75:**6187–6197.
20. **Bosilevac JM, Koohmaraie M.** 2011. Prevalence and characterization of non-O157 Shiga toxin-producing *Escherichia coli* isolated from commercial ground beef in the United States. Appl. Environ. Microbiol. **77:**2103–2112.
21. **Ammon A, Petersen LR, Karch H.** 1999. A large outbreak of hemolytic uremic syndrome caused by an unusual sorbitol-fermenting strain of *Escherichia coli* O157:H^-. J. Infect. Dis. **179:**1274–1277.
22. **Boczek LA, Johnson CH, Rice EW, Kinkle BK.** 2006. The widespread occurrence of the enterohemolysin gene *ehlyA* among environmental strains of *Escherichia coli.* FEMS Microbiol. Lett. **254:**281–284.
23. **Irino K, Vieira MA, Gomes TA, Guth BE, Naves ZV, Oliveira MG, dos Santos LF, Guirro M, Timm CD, Pigatto CP, Farah SM, Vaz TM.** 2010. Subtilase cytotoxin-encoding *subAB* operon found exclusively among Shiga toxin-producing *Escherichia coli* strains. J. Clin. Microbiol. **48:**988–990.
24. **Toma C, Martínez Espinosa E, Song T, Miliwebsky E, Chinen I, Iyoda S, Iwanaga M, Rivas M.** 2004. Distribution of putative adhesins in different seropathotypes of Shiga toxin-producing *Escherichia coli*. J. Clin. Microbiol. **42:**4937–4946.
25. **Paton AW, Srimanote P, Woodrow MC, Paton JC.** 2001. Characterization of Saa, a novel autoagglutinating adhesin produced by locus of enterocyte effacement-negative Shiga-toxigenic *Escherichia coli* strains that are virulent for humans. Infect. Immun. **69:**6999–7009.
26. **Jenkins C, Perry NT, Cheasty T, Shaw DJ, Frankel G, Dougan G, Gunn GJ, Smith HR, Paton AW, Paton JC.** 2003. Distribution of the *saa* gene in strains of Shiga toxin-producing *Escherichia coli* of human and bovine origins. J. Clin. Microbiol. **41:**1775–1778.
27. **Galli L, Miliwebsky E, Irino K, Leotta G, Rivas M.** 2010. Virulence profile comparison between LEE-negative Shiga toxin-producing *Escherichia coli* strains isolated from cattle and humans. Vet. Microbiol. **143:**307–313.
28. **Lucchesi PM, Krüger A, Parma AE.** 2006. Distribution of *saa* gene variants in verocytotoxigenic *Escherichia coli* isolated from cattle and food. Res. Microbiol. **157:**263–266.
29. **Paton AW, Beddoe T, Thorpe CM, Whisstock JC, Wilce MC, Rossjohn J, Talbot UM, Paton JC.** 2006. AB5 subtilase cytotoxin inactivates the endoplasmic reticulum chaperone BiP. Nature **443:**548–552.
30. **Cergole-Novella MC, Nishimura LS, Dos Santos LF, Irino K, Vaz TM, Bergamini AM, Guth BE.** 2007. Distribution of virulence profiles related to new toxins and putative adhesins in Shiga toxin-producing *Escherichia coli* isolated from diverse sources in Brazil. FEMS Microbiol. Lett. **274:**329–334.
31. **Michelacci V, Tozzoli R, Caprioli A, Martínez R, Scheutz F, Grande L, Sánchez S, Morabito S.** 2013. 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. Clin. Microbiol. Infect. **19:**E149–E156.
32. **Feng PCH, Councell T, Key C, Monday SR.** 2011. Virulence characterization of Shiga-toxigenic *Escherichia coli* serotypes isolated from wholesale produce. Appl. Environ. Microbiol. **77:**343–345.
33. **Kumar A, Taneja N, Kumar Y, Sharma M.** 2012. Detection of Shiga toxin variants among Shiga toxin-forming *Escherichia coli* isolates from animal stool, meat and human stool samples in India. J. Appl. Microbiol. **113:**1208–1216.
34. **Tasara T, Bielaszewska M, Nitzsche S, Karch H, Zweifel C, Stephan R.** 2008. Activatable Shiga toxin 2d (Stx2d) in STEC strains isolated from cattle and sheep at slaughter. Vet. Microbiol. **131:**199–204.
35. **Monday SR, Keys C, Hansen P, Shen Y, Whittam TS, Feng P.** 2006. Produce isolates of *Escherichia coli* Ont:H52 serotype that carry both Shiga toxin 1 and stable toxin genes. Appl. Environ. Microbiol. **72:**3062–3065.
36. **Beutin L, Miko A, Krause G, Pries K, Haby S, Steege K, Albrecht N.** 2007. Identification of human-pathogenic strains of Shiga toxin-producing *Escherichia coli* from food by a combination of serotyping and molecular typing of Shiga toxin genes. Appl. Environ. Microbiol. **73:**4769–4775.
37. **Brett KN, Ramachandran V, Hornitzky MA, Bettelheim KA, Walker MJ, Djordjevic SP.** 2003. *stx1c* is the most common Shiga toxin 1 subtype among Shiga toxin-producing *Escherichia coli* isolates from sheep but not among isolates from cattle. J. Clin. Microbiol. **41:**926–936.
38. **Friedrich AW, Borell J, Bielaszewska M, Fruth A, Tschape H, Karch H.** 2003. Shiga toxin 1c-producing *Escherichia coli* strains: phenotypic and genetic characterization and association with human disease. J. Clin. Microbiol. **41:**2448–2453.
39. **Schmidt H, Scheef J, Morabito S, Caprioli A, Wieler L, Karch H.** 2000. A new Shiga toxin 2 variant (Stx2f) from *Escherichia coli* isolated from pigeons. Appl. Environ. Microbiol. **66:**1205–1208.
40. **Persson S, Olsen KE, Ethelberg S, Scheutz F.** 2007. Subtyping method for *Escherichia coli* Shiga toxin (verocytotoxin) 2 variants and correlations to clinical manifestations. J. Clin. Microbiol. **45:**2020–2024.
41. **van Duynhoven YT, Friesema IH, Schuurman T, Roovers A, van Zwet AA, Sabbe LJ, Van Der Zwaluw WK, Notermans DW, Mulder B, vanHannen EJ, Heilmann FG, Buiting A, Jansen R, Kooistra-Smid AM.** 2008. Prevalence, characterization and clinical profiles of Shiga toxin-producing *Escherichia coli* in The Netherlands. Clin. Microbiol. Infect. **14:**437–445.
42. **Friedrich AW, Bielaszewska M, Zhang W-L, Pulz M, Kuczius T, Ammon A, Karch H.** 2002. *Escherichia coli* harboring Shiga toxin 2 gene variants: frequency and association with clinical symptoms. J. Infect. Dis. **185:**74–84.
43. **Garcia-Aljaro C, Muniesa M, Jofre J, Blanch AR.** 2006. Newly identified bacteriophage carrying the *stx2g* Shiga toxin gene isolated from *Escherichia coli* strains in polluted waters. FEMS Microbiol. Lett. **258:**127–135.
44. **Beutin L, Kruger U, Krause G, Miko A, Martin A, Strauch E.** 2008. Evaluation of major types of Shiga toxin 2e-producing *Escherichia coli* bacteria present in food, pigs and the environment as potential pathogens for humans. Appl. Environ. Microbiol. **74:**4806–4816.
45. **Bielaszewska M, Friedrich AW, Aldick T, Schurk-Bulgrin R, Karch H.** 2006. Shiga toxin activatable by intestinal mucus in *Escherichia coli* isolated from humans: predictor for a severe clinical outcome. Clin. Infect. Dis. **43:**1160–1167.
46. **Melton-Celsa AR, Kokai-Kun JF, O'Brien AD.** 2002. Activation of Shiga toxin type 2d (Stx2d) by elastase involves cleavage of the C-terminal two amino acids of the A2 peptide in the context of the appropriate B pentamer. Mol. Microbiol. **43:**207–215.