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Comparison of Shiga Toxin Production by Hemolytic-Uremic Syndrome-Associated and Bovine-Associated Shiga Toxin-Producing *Escherichia coli* Isolates

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There is considerable diversity among Shiga toxin (Stx)-producing *Escherichia coli* (STEC) bacteria, and only a subset of these organisms are thought to be human pathogens. The characteristics that distinguish STEC bacteria that give rise to human disease are not well understood. Stxs, the principal virulence determinants of STEC, are thought to account for hemolytic-uremic syndrome (HUS), a severe clinical consequence of STEC infection. Stxs are typically bacteriophage encoded, and their production has been shown to be enhanced by prophage-inducing agents such as mitomycin C in a limited number of clinical STEC isolates. Low iron concentrations also enhance Stx production by some clinical isolates; however, little is known regarding whether and to what extent these stimuli regulate Stx production by STEC associated with cattle, the principal environmental reservoir of STEC. In this study, we investigated whether toxin production differed between HUS- and bovine-associated STEC strains. Basal production of Stx by HUS-associated STEC exceeded that of bovine-associated STEC. In addition, following mitomycin C treatment, Stx2 production by HUS-associated STEC was significantly greater than that by bovine-associated STEC. Unexpectedly, mitomycin C treatment had a minimal effect on Stx1 production by both HUS- and bovine-associated STEC. However, Stx1 production was induced by growth in low-iron medium, and induction was more marked for HUS-associated STEC than for bovine-associated STEC. These observations reveal that disease-associated and bovine-associated STEC bacteria differ in their basal and inducible Stx production characteristics.

Shiga toxin-producing *Escherichia coli* (STEC) are emerging human pathogens that can cause diarrheal disease as well as more-severe clinical manifestations, including hemorrhagic colitis and hemolytic-uremic syndrome (HUS) (19, 46). The main environmental reservoirs of STEC are cattle and other ruminants, which often carry STEC among their intestinal floras (25); however, infected animals do not exhibit symptoms. Humans become infected with STEC primarily through ingestion of contaminated meat or other food products or by fecal-oral transmission of STEC from infected humans or animals (46). Only a subgroup of STEC within the bovine reservoir are thought to be capable of causing infection and disease (3). Evidence for this hypothesis includes differences in the prevalence of STEC serotypes isolated from cattle versus that of STEC serotypes isolated from infected humans (3, 25, 42). It is presently unclear whether serotype per se contributes directly to STEC pathogenesis (16) or whether other genetic differences account for the range of STEC pathogenicity (2).

STEC are mucosal pathogens that produce several virulence factors thought to contribute to their pathogenicity. Among these, the principal virulence factor is Shiga toxin (Stx), an A-B-type toxin that inhibits protein synthesis in target cells (reviewed in reference 48). Stx produced by STEC within the intestine is thought to enter the systemic circulation and result in damage to distal organs. Several observations suggest that the risk of serious complications of STEC infection (e.g., HUS) is correlated with the presence and amount of Stx produced during infection. First, intravenous administration of Stx to baboons resulted in microangiopathic hemolytic anemia (47), the key pathological feature seen in HUS (33). Second, according to the results of investigations with a mouse model, antibiotics that increase Stx production in the intestine can also increase mortality in mice infected with STEC (58). Similarly, administration of antibiotics to patients with STEC infection has been epidemiologically associated with more-severe clinical outcomes (55). The mechanism by which this occurs has yet to be elucidated; however, a strong possibility is that the antibiotics induced toxin production (17, 29, 58) and exacerbated the Stx-linked symptoms of STEC infection.

In addition to *stx*, several other genes are thought to contribute to STEC virulence. For example, genes within the locus of enterocyte effacement pathogenicity island are thought to mediate the adhesion of STEC to epithelial cells. In particular, the locus of enterocyte effacement gene *eae*, which encodes intimin, enables intimate attachment of bacteria to the gut epithelium (49; reviewed in reference 13). However, this gene is apparently not required for virulence, as *eae*-negative STEC have been isolated from patients with HUS (39). Additional potential virulence genes include an enterohemolysin, *ehxA* (also known as enterohemorrhagic *E. coli hlyA*) (4), a catalase-peroxidase (*katP*) (6), an extracellular serine protease (*espP*)

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(7), and a secreted metalloprotease, *stcE* (26). Of these, most is known about *ehxA*, the presence of which is associated with severe disease in humans (2, 5, 27, 45). The roles of these additional factors in STEC pathogenicity await additional confirmation.

Two main types of Stx are found in STEC, Stx1 and Stx2, each of which has one or more closely associated variant(s), namely Stx1c (57) and Stx2c, Stx2d, Stx2e, and Stx2f (14). The genes for these toxins are encoded in the genomes of bacteriophages related to phage lambda (20, 36, 38, 50, 54). Within these phage genomes, the *stx* genes are located between orthologues for the lambda genes *Q* and *S*, which are important for late gene transcription and lysis, respectively (23, 37, 41). Based on studies of relatively few disease-linked STEC isolates, it is thought that Stx production can be regulated by a number of mechanisms. Study of lysogens of the Stx1-encoding phage H-19B showed that low environmental iron concentrations can augment stx_1 transcription by relieving iron-dependent Fur-mediated repression at an stx_1 promoter (8). Stx2 production has not been found to be iron regulated; however, stx_2 expression can be induced by prophage-inducing stimuli, including the DNA-damaging agent mitomycin C (35, 56), H_2O_2 (52), and members of certain classes of antibiotics (17, 29). These stimuli induce the bacterial SOS response, which results in cleavage of the phage repressor, CI (43). Following cleavage of CI, the late phage genes, including *stx*, are efficiently transcribed and phage particles are produced. Recently, Wagner et al. demonstrated that expression of stx_2 under these circumstances is primarily dependent on initiation of transcription at the phage promoter $p_{R'}$ and upon antitermination by the antiterminator Q (54). stx_1 expression by H-19B is also induced by mitomycin C; however, $p_{R'}$ and Q appear to be less important in this strain (53).

Most previous comparative studies of human disease and bovine-linked STEC have focused on the distribution of virulence-associated genes rather than on production of the factors they encode. Since Stx production appears to be a critical determinant in the development of severe disease, we examined whether HUS- and bovine-associated isolates produced differing amounts of Stx. We compared levels of Stx production by these isolates in response to a variety of stimuli. The human isolates used for this study all came from nonepidemic HUS patients. We found that mitomycin C induced Stx2 production to higher levels in disease-associated isolates than in bovine-associated isolates. Similarly, low iron concentrations induced Stx1 production to a greater extent in disease-associated isolates. These findings suggest that environmental control of Stx production differs among disease-associated STEC isolates.

MATERIALS AND METHODS

Sample collection, isolation, and characterization of STEC. To obtain human disease-associated STEC isolates, stool samples were collected from 31 patients diagnosed with HUS during an ongoing study of the epidemiology of STEC in the United States. Each patient represented a sporadic case and was the source of only one STEC strain. In addition, six Stx1-producing STEC isolates from patients with HUS were kindly provided by H. Schmidt (Institut fur Hygiene und Mikrobiologie der Universitat Wurzburg, Wurzburg, Germany). STEC isolates with no known human disease association were isolated from bovine fecal samples obtained from cattle pastures in Nebraska; some fecal samples were taken from different pastures on the same farm, while other samples were obtained from geographically distant farms. Fresh fecal samples were collected and stored frozen until arrival at the laboratory.

Homogenized stool (or 25 ml of water in those situations in which we were testing water from the cattle drinking supply) was added to MacConkey broth, and the culture was incubated overnight with shaking at 37°C. The overnight culture was then screened for Stx production by enzyme immunoassay (Premier EHEC; Meridian Diagnostics Inc., Cincinnati, Ohio). Stx-producing single colonies were subsequently identified using a colony immunoblot assay (21). The presence of stx_1 and stx_2 genes was determined by PCR analysis using primers specific for the A subunit coding region as described by Paton and Paton (40). To simplify the interpretation of the results, only those isolates that contained either stx_1 or stx_2 (which represented 43% of the total number of isolates obtained), but not both, were used in subsequent experiments. Using PCR followed by digestion of the amplification product with *Hae*III and *Fok*I as described by Friedrich et al. (14), variants of the stx_2 gene were identified. The stx_{1c} variant was identified by PCR as described by Zhang et al. (57). Genes encoding intimin (*eae*) and enterohemolysin (*ehxA*) were detected by multiplex PCR essentially as described by Paton and Paton (40). In this assay, the primers used to detect *eae* are specific for the conserved region at the start of the gene; hence, both STEC and enteropathogenic *E. coli* intimin types are amplified. Typing for the *E. coli* O antigen was performed using standard methods (Mike Davis, *E. coli* Reference Laboratory, University Park, Pa.).

Mitomycin C induction experiments. Overnight cultures of each isolate were subcultured (5% inoculum) into two tubes containing 5 ml of fresh Luria broth and grown with shaking at 37°C to an optical density at 600 nm of 0.6. Mitomycin C (200 ng ml^{-1} final concentration) was added to one tube, while the second received an equivalent volume of distilled water. At 3 h after mitomycin C addition, 1 ml of culture was removed and sonicated (Sonifier 450; Branson Sonic Power, Danbury, Conn.) until cell lysis was evident (based on assessment of the extent of visual turbidity and confirmed by microscopic examination of selected samples). The total Stx concentration in each sample was then determined by an enzyme-linked immunosorbent assay (ELISA) as described previously (11). All the Stx1 and Stx2 variants identified in the STEC isolates studied here were detectable by the ELISA used in this study.

Influence of iron concentration on Stx1 production. A single colony from each isolate was inoculated into 2 ml of syncase broth (low-iron medium) (12) and grown overnight at 37°C with shaking. The overnight cultures were diluted to an optical density at 600 nm of 0.1 in fresh syncase medium or syncase medium supplemented with iron (10 μg of Fe [as $FeCl_3$] ml^{-1}) (iron-replete medium) and grown with shaking at 37°C for 3 h. The cultures were treated with polymyxin B (2 mg ml^{-1}) to release periplasmic Stx1 (10), and the total (periplasmic and extracellular) Stx1 concentrations were determined by ELISA. Polymyxin B was used instead of sonication in this experiment to facilitate rapid sample processing.

Statistical analysis. The median and the interquartile range (IR) of the Stx concentration obtained from each group are presented below. The IR describes the distribution of the central 50% of observations (i.e., those which are located between the 25th and 75th percentiles). The Wilcoxon rank-sum test was used to determine whether there were statistically significant differences between mitomycin C-treated and/or low-iron-induced total Stx1 or Stx2 concentrations produced by HUS- and bovine-associated STEC (9). All experiments were carried out in duplicate.

RESULTS

Characterization of Stx2-producing STEC isolates. A total of 13 Stx2-producing STEC isolates that contained amplifiable stx_2 but not stx_1 were obtained from stools of patients diagnosed with HUS. The majority of these isolates contained only stx_2, and two isolates contained both stx_2 and stx_{2c}. PCR assays revealed that all of these isolates contained amplifiable *eae* and *ehxA* sequences. The majority of these isolates (11 of 13) belonged to serogroup O157, while the remaining 2 isolates belonged to serogroup O121 (Table 1). This association of the O157 serogroup with HUS following infection with Stx2-producing STEC corroborates previous observations (18).

Twenty-five isolates of STEC producing only Stx2 were obtained from cattle pastures. One of these isolates was obtained from a water supply, while all of the others were isolated

TABLE 1. Characteristics of Stx2-producing HUS- and bovine-associated STEC isolates

Isolate	Source	Stx2 type(s)	Serogroup	Gene[a]		PCR product with primers for[b]:		Total [Stx2][e] (ng ml^{-1})
				eae	*ehxA*	*Q*-stx_2[c]	stx_2-*S*[d]	
H2A	HUS patient	2	O157	+	+	+	+	11,960
H2B	HUS patient	2, 2c	O157	+	+	+	−	26,230
H2C	HUS patient	2	O157	+	+	+	−	13,240
H2D	HUS patient	2	O157	+	+	+	−	12,840
H2E	HUS patient	2	O157	+	+	+	−	10,270
H2F	HUS patient	2	O157	+	+	+	+	11,160
H2G	HUS patient	2	O121	+	+	+	−	9,340
H2H	HUS patient	2	O157	+	+	+	−	9,650
H2I	HUS patient	2	O157	+	+	+	−	9,340
H2J	HUS patient	2	O121	+	+	+	−	9,050
H2K	HUS patient	2, 2c	O157	+	+	+	−	4,010
H2L	HUS patient	2	O157	+	+	+	−	10,870
H2M	HUS patient	2	O157	+	+	+	+	5,410
B2A	Cattle	2c	O6	−	−	+	+	170
B2B	Cattle	2c	O6	−	−	+	−	10
B2C	Cattle	2	O38	−	+	−	+	10
B2D	Cattle	2c	O91	−	−	−	−	200
B2E	Cattle	2, 2c	O96	−	+	−	+	880
B2F	Cattle	2	O98	+	+	−	+	10,190
B2G	Cattle	2	O113	−	−	−	+	10
B2H	Cattle	2, 2c	O113	−	+	+	+	100
B2I	Cattle	2, 2c	O113	−	+	−	+	520
B2J	Cattle	2c	O157	+	−	−	−	3
B2K	Cattle	2	O157	−	−	−	−	11,440
B2L	Cattle	2c	O157	−	−	−	+	30
B2M	Cattle	2	O163	−	+	−	+	20
B2N	Cattle	2, 2c	O165	+	+	−	+	2,520
B2O	Cattle	2c	O171	−	−	−	+	250
B2P	Cattle	2	O172	+	+	+	+	10,790
B2Q	Cattle	2	OX3[f]	−	−	−	−	980
B2R	Cattle	2c	OX3	−	−	−	+	130
B2S	Cattle	2	OX18	−	+	−	+	980
B2T	Cattle	2	OX25	−	+	−	+	17,110
B2U	Cattle	2c	NEG[g]	−	−	+	+	1,210
B2V	Cattle	2	NEG	−	−	−	+	4,990
B2W	Creek water	2	NEG	−	+	+	+	1,370
B2X	Cattle	2c	NEG	+	+	+	+	1
B2Y	Cattle	2, 2c	NEG	+	+	−	+	1,300

[a] +, presence of indicated gene; −, absence of indicated gene.
[b] +, presence of indicated PCR product; −, absence of indicated PCR product.
[c] PCR product size of 900 bp between *Q* and stx_2.
[d] PCR product size of 3,455 bp between stx_2 and *S*.
[e] Total Stx2 concentration in mitomycin C-treated culture (see text for details).
[f] OX serogroups refer to a provisional designation for an as-yet-unclassified O group.
[g] NEG, isolates did not react with any of the 181 O antisera used in standard testing.

directly from bovine feces. Of these 25 isolates, 11 contained stx_2, 9 contained only stx_{2c}, and 5 contained both stx_2 and stx_{2c} (Table 1). In contrast to the disease-associated STEC isolates, only 6 of the 25 bovine-associated STEC isolates contained amplifiable *eae* sequences and only 13 of the 25 contained amplifiable *ehxA* sequences. Also in contrast to the disease-associated isolates, there was a large range of O serogroups present in the bovine isolates (Table 1). Some isolates were classified as serogroup OX, which refers to a provisional designation for as-yet-unclassified or nonstandardized O-antigen groups. In addition, 5 of the 25 bovine-associated STEC isolates did not react to any of the 181 O antisera used in testing. The bovine-associated O157 isolates (three of three) were all *ehxA* negative, and two were *eae* negative. Since *eae* and *ehxA* are generally present in human O157 isolates (as noted above), additional tests were performed to confirm the species and serogroup classification of these three isolates.

Characterization of Stx1-producing STEC isolates. No HUS-associated STEC isolates producing Stx1 only were identified in the ongoing epidemiological study of STEC in the United States; consequently, Stx1-producing isolates from Germany were obtained. All these isolates contained stx_1 and not a recently identified variant, stx_{1c} (Table 2). The six Stx1-producing HUS-associated STEC isolates all contained amplifiable *eae* and *ehxA* sequences. However, unlike the Stx2-producing isolates, none belonged to serogroup O157; instead, they belonged to a range of serogroups including O103 and O111 (Table 2), which are the most common serogroups of Stx1-producing STEC isolates obtained from HUS patients (28).

TABLE 2. Characteristics of Stx1-producing HUS- and bovine-associated STEC isolates

Isolate	Source	*stx1* type	Serogroup	Gene		PCR product with primers for:		Total [Stx1][e] (ng ml^{-1}) in the presence of mitomycin C	Total [Stx1][f] (ng ml^{-1}) after growth in low Fe
				eae	*ehxA*	*Q-stx*$_1$[a]	*stx*$_1$-*S*		
H1A	HUS patient[g]	1	O26:H11	+	+	−	−	2,230	1,610
H1B	HUS patient[g]	1	O92:H33	+	+	+	+[c]	460	2,740
H1C	HUS patient[g]	1	O103:H2	+	+	−	−	370	5,020
H1D	HUS patient[g]	1	O103:H−	+	+	−	−	660	2,390
H1E	HUS patient[g]	1	O118:H−	+	+	+	−	2,430	1,550
H1F	HUS patient[g]	1	O145:H−	+	+	+	+[c]	1,170	1,320
B1A	Water sample	1	O103	+	+	+	−	210	13,160
B1B	Water sample	1	O103	+	+	−	−	180	14,910
B1C	Cattle	1	O109	−	+	+	−	7,690	1,930
B1D	Cattle	1	O109	−	+	−	−	110	30
B1E	Coyote[h]	1	O111	+	+	+	−	50	4,210
B1F	Cattle	1c	O121	−	−	−	+[b]	10	100
B1G	Cattle	1c	O121	−	−	−	−	210	120
B1H	Cattle	1	O141	−	+	+	−	<1	280
B1I	Cattle	1c	O142	−	−	−	+[b]	290	350
B1J	Water sample	1c	O142	−	−	−	−	<1	10
B1K	Cattle	1	O145	+	+	−	−	960	2,570
B1L	Cattle	1	O145	+	+	−	+[d]	60	2,400
B1M	Water sample	1c	NEG[i]	−	−	−	−	210	670
B1N	Cattle	1c	NEG	−	−	+	−	250	280
B1O	Cattle	1c	NEG	−	−	−	+[b]	1	30
B1P	Cattle	1	NEG	−	+	−	−	1,370	1,040
B1Q	Water sample	1	NEG	−	+	+	−	7,150	1,760
B1R	Cattle	1	NEG	+	+	+	−	220	10,310
B1S	Water sample	1	NEG	+	+	−	+[d]	120	3,290
B1T	Cattle	1	NEG	+	+	−	−	290	6,890

[a] PCR product size of 700 bp.
[b] PCR product size of 600 bp.
[c] PCR product size of 1,000 bp.
[d] PCR product size of 3,000 bp.
[e] Total Stx1 concentration following mitomycin C treatment.
[f] Values indicate total Stx1 concentration following growth in low-iron medium.
[g] Isolates obtained from H. Schmidt (Wurzburg, Germany). Serotypes for these isolates were provided by H. Schmidt.
[h] Fecal sample from coyote found in a pasture enclosure.
[i] Isolates did not react with any of the 181 O antisera used in standard testing.

A total of 20 Stx1-producing STEC isolates from cattle pastures were obtained from feces and water supplies and, in one instance, from coyote feces found in a pasture enclosure. Of the 20 isolates, 7 contained the *stx*$_{1c}$ gene. Similar to the bovine-associated Stx2-producing STEC, only 8 of the 20 Stx1-producing STEC isolates contained *eae* sequences and 13 of 20 contained *ehxA* sequences. In all 26 of the Stx1-producing STEC isolates investigated in this study, *eae* and *ehxA* status appeared to be serogroup specific. For example, all O103 and O145 isolates contained amplifiable *eae* and *ehxA* sequences whereas all O109 isolates contained amplifiable *ehxA* sequences only. The bovine-associated Stx1-producing isolates belonged to a diverse range of O-antigen groups, including O103, O121, and O142 (Table 2).

Mitomycin C-induced Stx production. There were marked differences in the effects of mitomycin C, an agent known to induce prophages, on Stx2 production by HUS- and bovine-associated isolates. Stx2 production by HUS-associated isolates increased dramatically during growth in mitomycin C; typically, at least 100-fold increases in Stx2 production were observed for these HUS-associated isolates (median fold increase [median] = 134; IR = 117 to 195) (Fig. 1A). In contrast, mitomycin C treatment of bovine-associated Stx2-producing isolates resulted in variable, but significantly lower ($P < 0.001$), induction of Stx2 production (median = 41; IR = 6 to 78). The median concentration of Stx2 after mitomycin C treatment was also significantly greater ($P < 0.001$) for HUS-associated STEC than for bovine-associated STEC (Table 1). Stx2 concentrations in non-mitomycin C-treated cultures were also significantly greater ($P < 0.01$) for HUS-associated STEC (median = 77; IR = 49 to 102) than for bovine-associated STEC (median = 14; IR = 2 to 46). Some bovine isolates were highly induced (>100-fold for 4 of 25 isolates) and/or produced high concentrations (>10,000 ng ml^{-1} for 4 of 25 isolates) of Stx2 following mitomycin C treatment. There was no correlation between highly mitomycin C-inducible Stx2 production by bovine-associated isolates and serogroup or *eae* or *ehxA* status. In contrast to the marked increases in Stx2 production by all HUS-associated O157 isolates, Stx2 production by each of the three bovine-associated O157 isolates was induced <50-fold by mitomycin C and two of the three isolates showed hardly any induction of toxin production. Thus, mitomycin C inducibility of Stx2 production is not an intrinsic feature of all O157 STEC.

In contrast to the dramatic induction of Stx2 production by mitomycin C, this prophage-inducing agent had minimal effect on Stx1 production by HUS-associated isolates (Fig. 1B). Fol-

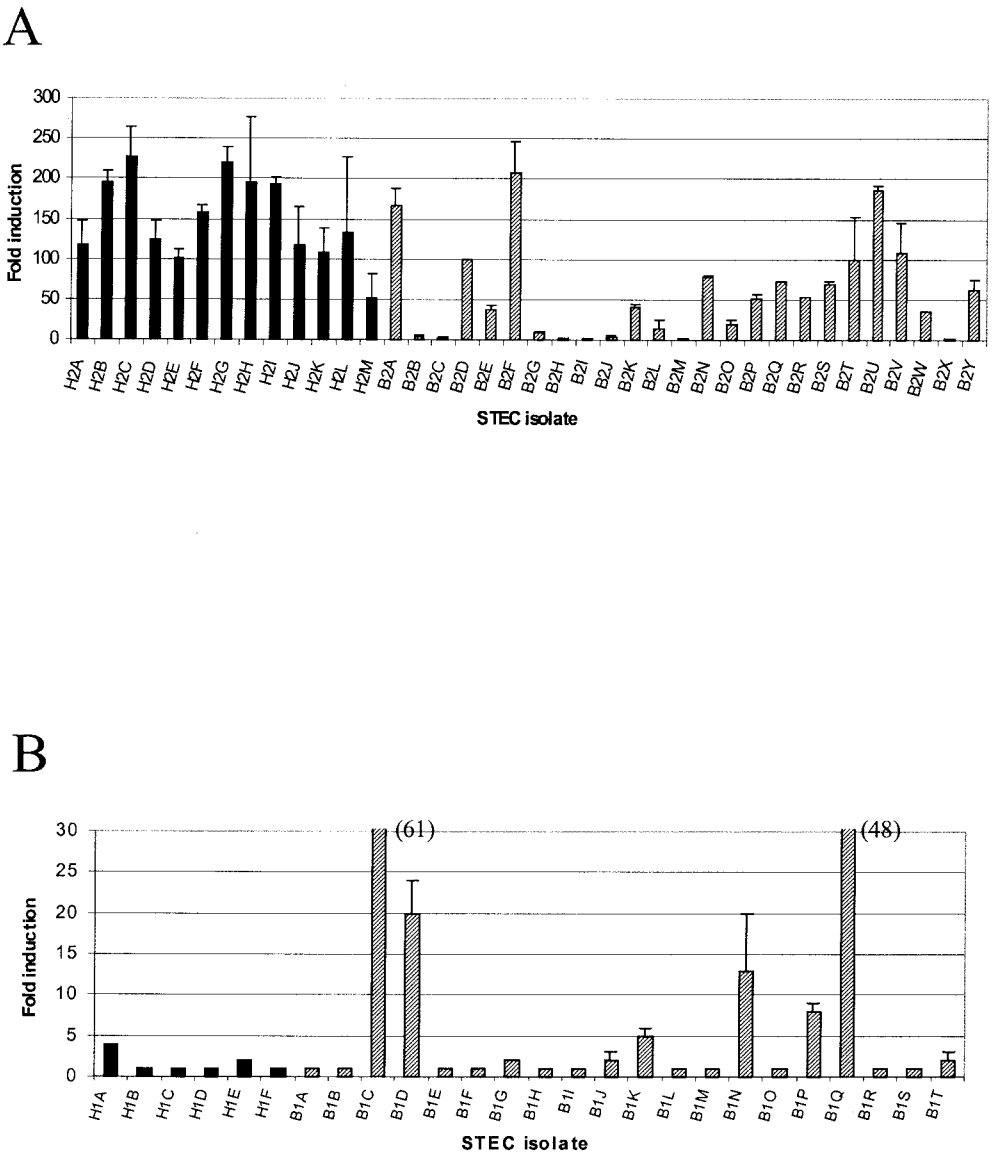

FIG. 1. Increase in Stx production following mitomycin C treatment of Stx2-producing (A) and Stx1-producing (B) HUS- and bovine-associated STEC. Each assay was performed on duplicate cultures; the mean and the standard deviation are presented for each isolate. The bars represent the fold change in toxin concentration of mitomycin C-treated cultures compared to that of the noninduced cultures, where fold change = [induced culture/uninduced culture]. Using this equation, a value of 1 signifies no change in Stx concentration. The median levels of induction of Stx2 production were 134- and 41-fold for HUS- and bovine-associated STEC isolates, respectively. The corresponding value for Stx1 production was 1 for both HUS- and bovine-associated STEC.

lowing mitomycin C treatment, Stx1 concentrations ranged from 300 to 2,400 ng of Stx1 ml^{-1} in cultures of these isolates (Table 2); in the majority, no increase (median = 1; IR = 1 to 1.5) in Stx1 concentration was observed. In the majority of bovine-associated Stx1-producing isolates (16 of 20), increases were less than 10-fold, although mitomycin C treatment of 4 isolates resulted in 13- to 60-fold increases in Stx1 production (Fig. 1B). Although the median fold increase in Stx1 production in response to mitomycin C treatment did not significantly differ between Stx1-producing HUS- and bovine-associated STEC isolates (Fig. 1B), there was a statistically significant difference ($P < 0.01$) in the total amounts of Stx1 produced following mitomycin C treatment of these two groups of STEC isolates (production of Stx by HUS isolates [median = 915; IR = 510 to 1,965] was greater than that by bovine isolates [median = 210; IR = 58 to 416]). This discrepancy reflects the fact that in the absence of mitomycin C, the HUS-associated isolates produced significantly greater ($P < 0.001$) amounts of Stx1 than bovine isolates; without addition of mitomycin C, the median Stx1 concentrations were 860 (IR = 660 to 1,110) and 120 (IR = 20 to 170) ng of Stx1 ml^{-1}, respectively, for these two groups.

Association of *stx* and bacteriophage genes. Since we found that mitomycin C treatment did not increase Stx production in all STEC isolates and previous experiments had shown that mitomycin C can induce toxin production by lysogens of both

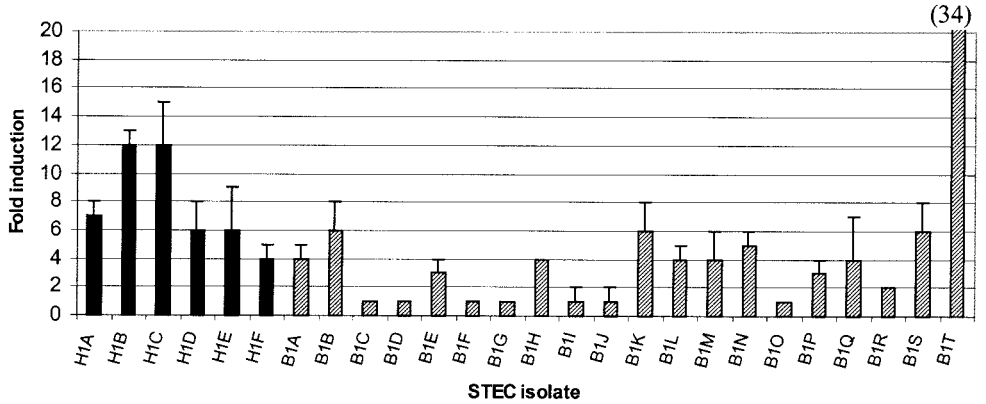

FIG. 2. Increase in Stx1 production following growth in low-iron medium of Stx1-producing HUS- and bovine-associated STEC. Each assay was performed on duplicate cultures; the mean and the standard deviation are presented for each isolate. The bars represent the difference in toxin concentration of cultures grown in low-iron medium compared to that of cultures grown with added iron (10 μg ml^{-1}). The fold change = [low iron/added iron]. Using this equation, a value of 1 signifies no change in Stx1 concentration. The median levels of induction were 6.5 and 3.5 for HUS- and bovine-associated STEC isolates, respectively.

Stx1- and Stx2-encoding phages (51), we used PCR to assess whether the *stx* genes studied here were located in phage genomes. Primers were designed based on the sequences of the Stx1-encoding phage H-19B or the Stx2-encoding phage 933W, as described previously (50). These primers amplify the segments between *Q* and *stx* and between *stx* and *S*. While the *stx* genes of all 13 HUS-associated Stx2-producing STEC were found adjacent to 933W-like *Q* sequences, linkage to a 933W-like *S* sequence was observed in only 3 (Table 1). Conversely, the *stx* genes in only 7 of 25 bovine-associated Stx2-producing STEC isolates were adjacent to the 933W-like *Q*, whereas the *stx* genes were linked to the 933W-like *S* sequence in a greater proportion of these isolates (20 of 25). These results reflect the diversity and likely mosaicism present in these stx_2-encoding phages. Overall, of the eight Stx2-encoding STEC isolates in which Stx2 production did not increase (defined as <10-fold) in response to mitomycin C treatment, seven contained stx_2 genes demonstrated by PCR to be linked to phage sequences. The *stx* genes of only three of six Stx1-producing HUS-associated STEC isolates were linked with H-19B-like sequences (*Q* or *S*; Table 2). Of 20 Stx1-producing bovine-associated isolates, linkage between the *stx* genes and H-19B-like *Q* or *S* sequences occurred in only 7 or 5, respectively. However, failure to detect the presence of neighboring phage genes did not always correspond with a lack of mitomycin C inducibility nor was detection of phage genes always accompanied by mitomycin C inducibility. Overall, the primary conclusion that can be drawn from these PCR assays is that although the majority of STEC isolates studied had *stx* genes flanked by phage sequences, the sequences surrounding some *stx* genes in STEC isolates were variable and appeared to contain sequences that differ from those of H-19B or 933W.

Low-iron-induced Stx1 production. Since Stx1 production had been shown previously to be induced when lysogens of H-19B were grown in low-iron medium, we compared levels of Stx1 production by HUS- and bovine-associated isolates to determine whether regulation of Stx1 production by iron concentration differs in these groups. The median fold increase in Stx1 production in low-iron medium was significantly greater ($P < 0.01$) in HUS-associated isolates (median = 6.5; IR = 6 to 9) than in bovine-associated STEC isolates (median = 3.5; IR = 1 to 5) (Fig. 2). However, note that Stx1 production by bovine-associated isolates was highly variable in both low- and high-iron media; some isolates produced high levels of Stx1 (>10,000 ng of Stx1 ml^{-1}). In contrast, there was less variability in Stx1 production by HUS-associated isolates grown in low- and high-iron media. Overall, levels of total Stx1 production following growth in low-iron medium did not differ significantly between the two groups. No correlation was observed between isolates in which Stx1 production was highly induced by growth in low-iron medium and those in which Stx1 production was induced by mitomycin C (Table 2).

DISCUSSION

Regulation of virulence factor production in response to environmental stimuli is thought to be a critical feature of microbial pathogenicity (32). Previous studies have documented how prophage-inducing stimuli (e.g., mitomycin C) and low-iron conditions can greatly augment Stx production (1, 15, 51, 53). However, these studies analyzed a limited number of human STEC isolates, and until now, little information existed regarding how bovine-associated STEC isolates respond to these stimuli. In this study, we found that not all STEC respond similarly, as marked differences in Stx production were found between HUS- and bovine-associated STEC isolates. The augmentation of Stx production by the presence of either mitomycin C or low iron concentration was, in general, significantly greater in HUS-associated STEC isolates than that observed for bovine-associated STEC isolates. That a subgroup of STEC can be more virulent for or more readily transmitted to humans was previously suggested by detection of genotypic variations in bovine and human O157 isolates (24, 31). Our results suggest that response to prophage-inducing stimuli or low iron contributes to the ability of a subgroup of STEC to cause severe disease in humans.

Among the HUS isolates, mitomycin C-inducible toxin production was detected in all the Stx2-producing isolates tested

but, unexpectedly, this prophage-inducing agent did not augment toxin production by Stx1-producing isolates. Previous work (for examples, see references 51, 53, and 54) has shown that this stimulus induces lysogens carrying either stx_1 or stx_2 to increase toxin synthesis, as it promotes phage transcription and replication. Our PCR analyses revealed that stx_1 was flanked by *Q* and/or *S* sequences in three of six of the HUS-associated isolates we studied; thus, these isolates might have been expected to be inducible with mitomycin C. Presumably, in these cases the stx_1 genes lie within defective prophages. In this regard, they may be like the *stx* genes in *Shigella dysenteriae* type 1, which have been shown to be adjacent to phage-like sequences (30) but are not transmissible.

Our results extend previous observations that there is a considerable diversity of *stx*-containing prophages (22, 50, 51). Both the range of augmentation of Stx production in response to mitomycin C treatment and the differing sizes of the PCR products amplified from the toxin-linked phage sequences are reflections of this heterogeneity. Another indication of this variability was the finding that Stx2 production in bovine-associated STEC was augmented by provision of the H-19B-like *Q* in *trans* in only a subset of the isolates tested (data not shown).

Our observations suggest that prophage induction is not as important for Stx1 production as it is for Stx2 production, since, as mentioned above, the HUS-associated Stx1-producing isolates we tested did not respond to mitomycin C treatment. The nature of the process by which Stx1 is released from bacteria in the absence of prophage induction is unclear, since phage-induced bacterial lysis is the only cellular program shown (at least in vitro) to allow Stx to escape from the periplasm (53). Our present data raise the possibility that additional processes also enable toxin release (at least from HUS-associated Stx1-producing strains). However, the apparent absence of prophage regulation of Stx1 production might account for the fact that Stx2-producing STEC isolates, either alone or in combination with Stx1, are more frequently associated with HUS than isolates that produce Stx1 alone (18). Alternatively, it is possible that some Stx-encoding phages are induced in vivo via pathways distinct from the SOS response (44). Overall, further study of prophage induction in vivo is warranted to determine whether host responses (e.g., H_2O_2 production by neutrophils) (52) have a significant impact or whether exogenous stimuli (e.g., antibiotic treatment) are the dominant modulators of toxin production.

The median levels of Stx production following growth in Luria broth also differed between the HUS-associated and bovine-associated STEC isolates we studied. This basal Stx production by both Stx1- and Stx2-producing HUS-associated isolates was significantly greater than that by bovine isolates. In particular, basal Stx production was high in Stx1-producing HUS isolates. This finding, coupled with the fact that prophage-inducing stimuli had a less marked effect on Stx1-producing isolates than on the Stx2-producing HUS isolates, suggests that Stx1 inducibility is not critical for the pathogenicity of these strains.

Factors other than basal and inducible Stx production are also important in STEC pathogenesis. As previous analyses have suggested (2), we found that *ehx* and *eae* are likely to be important contributors to STEC pathogenicity. Both genes were present in all HUS-associated STEC isolates analyzed. In contrast, only 13 of 45 of the bovine-associated STEC isolates contained both genes and 18 of 45 contained neither gene. However, neither *ehxA* nor *eae* appears to be essential for STEC pathogenicity, since clinical isolates lacking either *ehxA* or *eae* have been reported (34, 39). Still, these virulence factors appear to be more critical than serogroup for STEC pathogenicity; we found that HUS-linked isolates were from a variety of serogroups. Future studies of these factors should include quantitation of virulence gene expression, since we found that quantifying Stx production revealed a previously unrecognized distinction between disease- and bovine-associated STEC.

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