

Enhancement of Shiga Toxin Production in Enterohemorrhagic *Escherichia coli* Serotype O157:H7 by DNase Colicins[∇]

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Received 13 June 2007/Accepted 1 October 2007

Colicins are proteins produced by and active against several strains of *Escherichia coli*. Previously we reported that colicinogenic bacteria seemed beneficial in preventing the clinical manifestations of infectious disease caused by enterohemorrhagic *E. coli* O157 in humans. The inhibitory effects could be due to a decrease in O157 levels and/or pathogenicity. This study investigated the effects of colicinogenic *E. coli* on the production of Shiga toxin (Stx) by O157. Standard strains of colicinogenic bacteria carrying plasmids for each type of colicin (E3/5/8/9) were used for the study. The O157 strains were cultured in the presence of colicinogenic bacteria or extracted colicins. Compared with results for controls, DNase colicins (E8/9) facilitated an 8- to 64-fold increase in production of Stx2, while RNase colicins (E3/5) suppressed Stx production in only two strains. Stx prophages were induced in synchrony with Stx production. Semiquantitative real-time reverse transcription-PCR (RT-PCR) was then performed to examine SOS gene expression. The RT-PCR results clearly indicated a marked increase in mRNA levels of SOS reaction-associated genes after the addition of DNase colicins. We believe that Stx prophages are induced by the SOS response to DNA damage caused by DNase colicins, thus leading to higher Stx production. These findings suggest that while colicinogenic bacteria can be antagonistic to O157 infection, DNase colicins may enhance Stx production. Thus, colicinogenic flora is likely to be involved in the complex pathogenic pathways of O157 infection, and further investigation should be performed before the use of colicinogenic bacteria as an intervention method.

Escherichia coli is one of the predominant facultative anaerobes in the normal colon flora. However, particular clones can cause sepsis, urinary tract infection, and diarrheal disease. Diarrheagenic *E. coli* is classified according to pathogenicity as enteropathogenic *E. coli*, enterotoxigenic *E. coli*, enteroinvasive *E. coli*, enterohemorrhagic *E. coli* (EHEC), enteroaggregative *E. coli*, or diffusely adherent *E. coli* (17).

E. coli O157:H7 (O157), a serotype of EHEC, is infectious to humans at very low doses and is currently one of the most devastating food-borne pathogens in many parts of the world. EHEC is characterized by its ability to produce Shiga toxin (Stx), which is a major virulence factor in infectious disease (7). The Stx family is composed of two main groups, Shiga toxin 1 (Stx1) and Shiga toxin 2 (Stx2), with Stx2 apparently more virulent in humans than Stx1 (19). Both toxins are normally encoded in the genomes of lysogenic lambdoid phages. Stx causes apoptosis in intestinal epithelial cells and mediates local damage in the colon, thus resulting in hemorrhagic colitis. Furthermore, the toxin produced in the colon travels to the kidney and possibly to the brain via the bloodstream (3), which results in renal damage, leading to hemolytic-uremic syndrome

(HUS) and fatal neurological abnormalities. However, different individuals respond to exposure to EHEC in different ways, and it is not clear why some people are asymptomatic whereas others develop mild gastrointestinal illness to hemorrhagic colitis and still others develop full-blown HUS.

Normal gastrointestinal flora has marked effects on the morphological, physiological, and immunological development of the host (1). Resident microbiota play an important role in the prevention and/or amelioration of intestinal infections through a variety of mechanisms, such as (i) direct inhibition of pathogens by bacteriocins and/or metabolites, (ii) competition for receptors and nutrients, and (iii) stimulation of innate or acquired host immunity (12). Some strains have been used as probiotic agents based on the principle of competitive exclusion or displacement of diarrheagenic bacteria with nonpathogenic strains (20).

Bacteriocins are bacterial proteins that are lethal to other members of the same or closely related species. The bacteriocins produced by and active against *E. coli* and related bacteria are called colicins. Indigenous bacteria producing colicins are assumed to have a competitive advantage over nonproducing strains since the toxin acts directly against susceptible bacteria. Some colicinogenic *E. coli* strains have been reported to be effective in inhibiting diarrheagenic *E. coli*, including O157, in vitro (5, 15, 24, 25). Zhao et al. (33) has suggested that competitive exclusion of O157 may occur in cattle with administration of colicinogenic *E. coli* strains. Furthermore, we found that colicinogenic bacteria able to suppress the growth of O157 are more prevalent among healthy O157 carriers than among age-matched subjects who do not carry O157 (28). It thus

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[∇] Published ahead of print on 12 October 2007.

appears that inhibitory bacteria may play a role in inhibiting propagation of O157 and/or suppressing expression of virulence factors by this pathogen in the human gut.

Although colicinogenic bacteria are apparently useful candidates for the prevention of infectious disease caused by O157, it is known that some antibiotics, such as fluoroquinolones, accelerate Stx production in O157 (13, 27, 32). The production of Shiga toxins is one of the most critical factors in the development of HUS. It is probable that the more toxin produced, the more likely it is that a patient will develop significant medical problems. Since colicins are a type of antimicrobial agent, the purpose of this investigation was to examine whether colicinogenic bacteria or colicins influence the production of Stx. The RecA protease, a component of the so-called SOS system, causes phage induction and increased toxin production via concomitant multiplication of toxin gene copies. To elucidate the mechanism by which colicin elicits such effects, expression of SOS reaction-associated genes was also examined by semiquantitative real-time reverse transcriptase PCR (RT-PCR).

MATERIALS AND METHODS

Strains and cultures. The 10 strains of *E. coli* O157:H7 used in this study are all original isolates from our previous studies (18, 19, 28). Strain 96-98-83 was isolated from a diarrheal patient involved in the massive outbreak in Sakai City in 1996 (14), and strains 30723 and 20517 were from healthy carriers. These three strains produced both Stx1 and Stx2. Strain 0001 was isolated from a sporadic case and was shown to possess genes coding for Stx1 and a variant of Stx2 (Stx2vha) according to the scheme of Tyler (29). Stx2-producing strains 99-75, 2052, and 3001 were isolated from sporadic cases. Strains 0046 and 2015 were isolated from healthy carriers and possessed only Stx2vha. Strain 96-98-83/1 was a derivative of strain 96-98-83 that was found to have spontaneously lost the Stx2 prophage. Standard strains carrying colicin plasmids were used for preparation of colicins (28). Strains RR1-E3, RR1-E5, RR1-E8, and RR1-E9 produced the rRNase colicin E3, the tRNase colicin E5, the DNase colicin E8, and the DNase colicin E9, respectively. For a negative control, strain RR-1 that was not transformed with colicin plasmids was used in some experiments.

Bacteria were routinely grown at 37°C in Luria broth (L broth) (Difco Laboratories, Detroit, MI) or tryptone soya agar (Oxoid, Basingstoke, Hampshire, United Kingdom). Sorbitol-tryptone soya agar, which is tryptone soya agar supplemented with sorbitol (10 g/liter) and neutral red (0.03 g/liter; Sigma-Aldrich, St. Louis, MO), was used for differential counting of sorbitol-fermenting colicinogenic bacteria and nonfermenting O157 colonies; MacConkey sorbitol agar was not used, since it gave lower counts than sorbitol-tryptone soya agar.

Coculture. O157 strains or colicinogenic strains grown in L broth at 37°C were washed with phosphate-buffered saline (PBS) and resuspended in fresh L broth. Bacterial suspensions were prepared at a concentration of ca. 5×10^8 CFU/ml. Stx production was induced by coculture of O157 with colicinogenic strains in 10 ml of L broth. As a positive control, mitomycin C was added to another O157-inoculated culture at 250 ng/ml. The negative control was cultured without colicinogenic strains or mitomycin C. O157 was inoculated for culture at a concentration of 3×10^6 CFU/ml and was subsequently incubated at 37°C without shaking. At 24 h and 48 h, samples were taken from each tube and CFU and Stx concentrations were determined. For Stx assay, part of each sample was immediately frozen at -80°C. Viable counts (CFU/ml) were determined by the spiral plating method (Eddy Jet; IUL, Barcelona, Spain).

Colicin preparation. The method of Pugsley and Oudega (22), with some modifications, was used to prepare colicin solution. Colicinogenic strains were cultured in L broth overnight, and 5 ml of the culture was inoculated into 50 ml of fresh L broth. Organisms were then cultured at 37°C for 3 to 4 h with shaking (160 rpm). When the optical density at 600 nm reached at 0.4, mitomycin C solution (250 µg/ml) was added to the cultures at a final concentration of 500 ng/ml. After 1 h of additional incubation, culture broth was centrifuged for 10 min at $10,000 \times g$. Pelleted bacteria were resuspended in 5 ml of PBS and were disrupted by sonication (UH-50; SMT Co. Ltd., Tokyo, Japan). After centrifugation at $10,000 \times g$ for 10 min, the supernatant was filtrated through low-protein-binding 0.45-µm-pore-size membrane filters (Millex-HV; Millipore, Ireland) and was stored at -80°C as colicin solution. A standard strain that was not

transformed with colicin plasmids was used to prepare negative controls in some experiments. One arbitrary unit (AU) was defined as the reciprocal of the highest dilution that gave rise to a discernible zone of inhibition, as described below.

Antibacterial assay. The antibacterial activity of colicin solution was assayed by dropping 5 µl of colicin solution onto 5 ml of soft agar (1%) preseeded with 10^6 O157 cells on a regular-strength Luria agar plate, with 20 strains being spotted on each plate. Plates were then incubated overnight at 37°C, and drops showing zones of inhibition were considered to be putative colicin solution able to suppress O157 growth.

Effects of colicins on Stx production and induction of prophages. O157 strains grown in L broth at 37°C were washed with PBS and resuspended in fresh L broth. An aliquot of 100 µl of bacterial suspension was inoculated into 10 ml of fresh L broth to give an initial concentration of O157 of 1×10^6 CFU/ml, and then 100 µl of colicin solution or mitomycin C (final concentration, 250 ng/ml; positive control) was added. These samples were incubated statically at 37°C. Numbers of CFU, Stx concentrations, and numbers of PFU were determined at 6 h. A portion of each sample was immediately frozen at -80°C for subsequent Stx assays.

Viable counts, Stx assay, and phage plaque assay. Differential amounts of Stx1 and Stx2 were determined by a reverse passive latex agglutination test using VTEC-RPLA (Denka Seiken Co., Tokyo, Japan), as reported previously (19). To induce bacterial lysis, polymyxin B solution (5 mg/ml) was added at 100 µg/ml and was incubated at 37°C for 30 min. After centrifugation, twofold serial dilutions of the supernatant were prepared using 96-well microdilution plates, and these were mixed with solutions of latex particles coated with anti-Stx1 or anti-Stx2 monoclonal antibody. The reciprocal of the highest dilution causing latex agglutination was recorded as the titer. Stx levels were recorded as the toxin titer since the detection limit of the kit was about 1 ng/ml.

The number of phages released after prophage induction was determined by plaque assay. O157 culture broths were centrifuged, and the supernatants were filter sterilized through a 0.45-µm filter. The resulting filtrates containing free phage particles were serially diluted 10-fold and were then mixed with a suspension of indicator strain in L soft agar (0.5%) supplemented with 10 mM CaCl₂ and poured onto L agar plates; the *E. coli* K12 colicinogenic standard strain RR1-E9 was used as an indicator since it would likely possess resistance to contaminating colicins introduced with the samples. After overnight incubation at 37°C, the number of plaques formed in the top layer (PFU) was determined. The plaques (at least 20 plaques on each plate) were examined for Stx genes by using a PCR assay (4).

RNA extraction. Optical densities of bacterial cultures were measured at 600 nm, and their concentrations were adjusted. Culture broth (0.5 ml) containing 5×10^8 cells was mixed with 1 ml of RNAlater bacterial reagent (QIAGEN, Hilden, Germany) to stabilize the mRNA, and the mixture was stored at -80°C. Total RNA was subsequently extracted using the RNeasy minikit (QIAGEN), and contaminating DNA was digested using DNase I (Promega, Madison, WI) according to the manufacturer's protocol. The RNA sample was quantitated based on absorption at 260 nm, and purity was determined by the ratio of absorption values at 260 and 280 nm (GeneQuant *pro* S²RNA/DNA Calculator; Amersham Pharmacia Biotech, Uppsala, Sweden). Extracted RNA specimens were subjected to reverse transcription using the pd(N)₆ random hexamer (Amersham Pharmacia Biotech) and the Omniscript reverse transcriptase kit (QIAGEN) according to the manufacturer's protocol, and cDNA was used as a template for RT-PCR.

RT-PCR. PCR mixtures consisted of 0.1 µl of Ex Taq (Takara-Bio; Ohtsu, Shiga, Japan), 2 µl of 2.5 mM deoxynucleoside triphosphate mixture, 2 µl of 25 mM MgCl₂, 2.5 µl of 10× Ex Taq buffer, 0.1 µl of each primer at 50 µM, 1 µl of cDNA, and 17.2 µl of sterilized molecular-grade water. DNA extracted from an O157 strain (no. 3001) by boiling was used as a positive control. RNA samples that were not reverse transcribed were used as negative controls in order to ensure that amplification was due to cDNA templates and not contaminating genomic DNA. PCR was performed under the following conditions: denaturation at 95°C for 10 min and 30 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s, followed by a 5-min extension at 72°C and holding at 4°C. Primer sets are listed in Table 1. PCR products were separated on 2.5% agarose gels, followed by staining with ethidium bromide, and were visualized on a UV transilluminator.

Semiquantitative real-time RT-PCR. Relative expression values of SOS reaction-associated genes were measured, including a standard curve analysis for each gene. Semiquantitative real-time PCR was performed with QuantiTect SYBR green (Qiagen) according to the manufacturer's protocol on a PRISM 7000 sequence detection system (Applied Biosystems, Foster City, CA). Primers are shown in Table 1. PCR was performed in a 25-µl volume containing 200 µM deoxynucleoside triphosphates, 2.5 U of polymerase, 3 mM MgCl₂, 0.3 µl of each

TABLE 1. PCR conditions and primers used in this study

Target	Primer (5'→3') ^a	Product size (bp)	Reference
<i>recA</i>	f: AGCTACAAAGGTGAGAAGATCG r: TTCGCTATCATCTACAGAGAAATCC	158	22
<i>lexA</i>	f: GCATATTGAAGGTCATTATCAGGT r: ACCGTTACGTACATCCTGAGTTTT	153	22
<i>recN</i>	f: GTACAGCTGTTCTCTGTCAACAAC r: GTCATTTCTGCAGTAGAGAGGTT	170	22
<i>sulA</i>	f: CAACTTCTACTGTTGCCATTGTTAC r: GCTGGCTAATCTGCATTACTT	144	22
<i>yebG</i>	f: CGAAGAGAAAATGTCGTTTACCAG r: CTCAGCACATCTTTTGTCTGC	167	22
<i>dinI</i>	f: AGTATGCGTTTCTGTATAATGAAGG r: TATTCGCTGACAAACCAGTCAT	156	22
<i>uvrA</i>	f: ATAAAGTGGTGTGTACGGTTCTG r: CACGGACGATTACTGATAAACTTG	186	22
<i>uvrB</i>	f: GTTCCACTATTCCACGTTTACC r: GTAGTTTTCAATCCCCGAACAGTA	224	22
<i>cho</i>	f: GTGGTACGGCGTTAACTTCTC r: GTTAACGCTTTTCCGATATAGAG	143	22
<i>stx2</i>	f: ATTAACCACACCCACCG r: GTCATGGAAACCGTTGTCAC	200	4
<i>gapA</i>	f: TATGACTGGTCCGTCTAAAGACAA r: GGTTTTCTGAGTAGCGGTAGTAGC	201	22

^a f, forward; r, reverse.

primer at 50 μ M, and 2 μ l of genomic DNA. A batch of DNA template extracted from strain no. 3001 using the Cell and Tissue DNA Isolation kit (Gentra Systems, Minneapolis, MN) was used to produce serial dilutions, and amplified products from this dilution series were used to make a standard curve with which

to quantify the relative amounts of product in each experimental sample. Reaction mixtures were dispensed into a 96-well, thin-wall PCR plate (Applied Biosystems), covered with optically clear sealing film, and centrifuged briefly. PCR was performed under the following cycle conditions: denaturation at 95°C for 15 min and 40 cycles of 94°C for 15 s, 58°C for 30 s, and 72°C for 30 s, followed by a 5-min extension at 72°C and holding at 4°C. The threshold cycle for each standard was plotted against the log of starting quantities to construct a standard curve in order to quantify genes in unknown samples. To account for variations in the efficiency of the reverse transcription reactions between samples, we performed RT-PCR for the constitutively expressed *gapA* (D-glyceraldehyde-3-phosphate dehydrogenase) gene and normalized gene expression values detected in each sample against the values determined for *gapA*, as described previously (23). Each experiment was carried out in triplicate, and thus, the average of relative gene expression values was used to determine whether the expression values of a given gene significantly differed between the colicin-treated group and the mitomycin C-treated group. Because agarose gel electrophoresis of the amplicon produced by *lexA* primers showed some nonspecific bands, its use was precluded in semiquantitative real-time RT-PCR.

Statistics. Post hoc multiple comparisons between groups were made by using Tukey's test with the statistics add-in software Statcel 2 (OMS, Tokorozawa, Japan) for Microsoft Excel unless otherwise stated.

RESULTS

Effects of coculture with colicinogenic bacteria on Stx production. *E. coli* O157:H7 strain 96-98-83, which was the causative agent of the massive outbreak in Sakai City, Japan, in

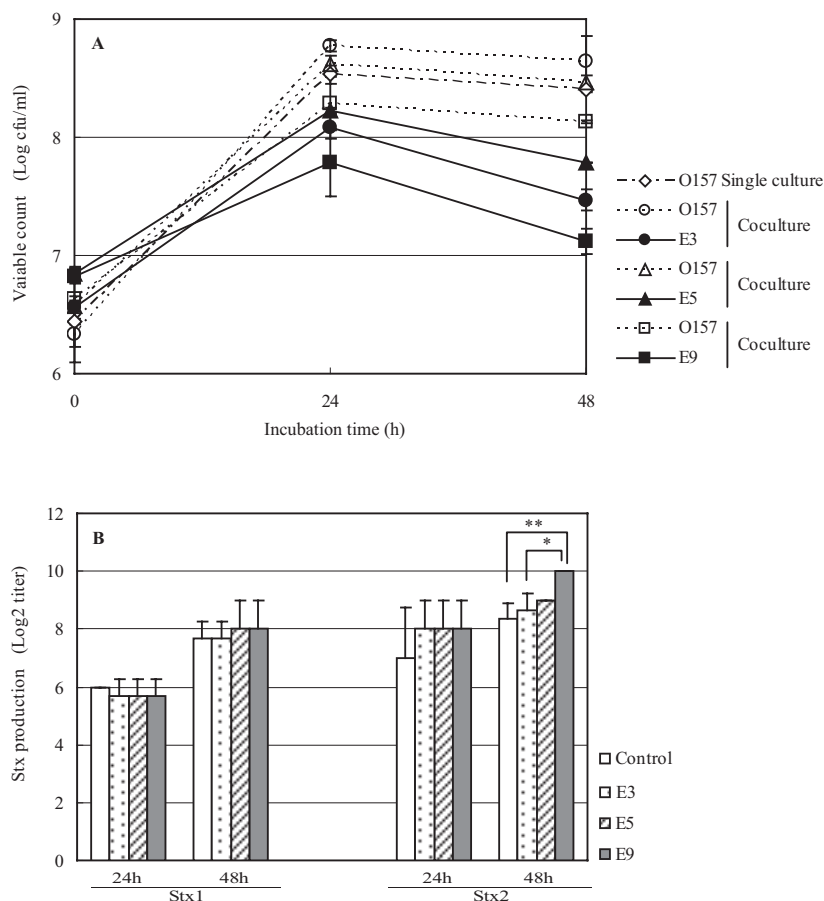


FIG. 1. Effects of coculture with colicinogenic bacteria on Stx production. *Escherichia coli* O157 strain 96-98-83 was cultured in the presence of colicinogenic bacteria. (A) After 24 h or 48 h of incubation, the viable count was determined. Coculture (full line) with *E. coli* O157 and the colicin E3 strain, with *E. coli* O157 and colicin E5, or with *E. coli* O157 and colicin E9 is shown. Single culture of *E. coli* O157 acted as a control. (B) Stx production was measured by using a reverse passive latex agglutination test. The reciprocal of the highest twofold serial dilution causing latex agglutination was recorded as the titer and is expressed as a binary logarithmic value. Data (means \pm standard errors) were from three independent experiments.

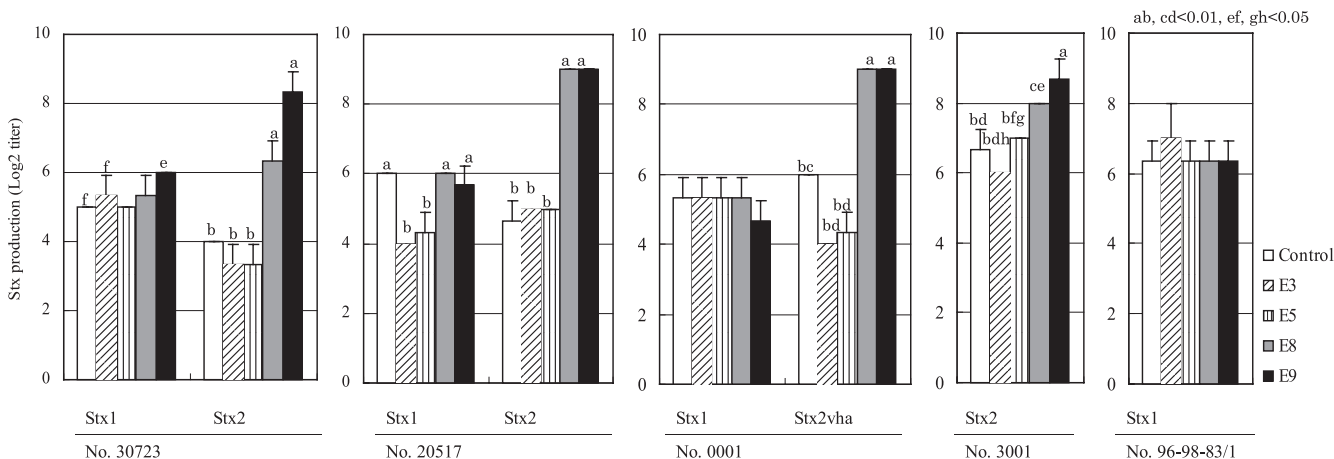


FIG. 2. Effects of extracted colicins on Stx production from several *Escherichia coli* O157 strains. *E. coli* O157 strains were incubated with 800 AU of each colicin. A standard strain that was not transformed with colicin plasmids was used to prepare negative controls. After 24 h of incubation at 37°C, concentrations of Stx were measured by using a reverse passive latex agglutination test. Data are expressed as multiples of Stx concentrations in the control. Ten strains were assayed three times, and data presented are from five strains possessing different combinations of Stx genes. Data (means ± standard errors) were from three independent experiments. Significant differences were observed at *P* values of <0.01 (between a and b or c and d in each group) or *P* values of <0.05 (between e and f or g and h in each group).

1996, was cocultured with colicin (E3, E5, or E9) producers at various concentration ratios. The 0-, 24-, and 48-h cultures were examined for viable cell counts, and the 24- and 48-h cultures were examined for Stx production. When the concentration ratio of colicin E9-producing bacteria to O157 was 1:1, the number of O157 bacteria was lower than that in single culture of O157 (Fig. 1A). However, Stx2 production was higher in coculture with the colicin E9 producer than in single culture (Fig. 1B). In contrast, with colicin E3 (rRNase) or E5 (tRNase) producers, no increase in Stx production was detected at 48 h. When the ratio of the E9 producer to O157 was 1:100 or 100:1, Stx production levels were similar to those in single culture of O157 (data not shown).

Effects of colicins on Stx production. In order to investigate whether the increase in Stx production was due to colicin produced by colicinogenic strains, the amount of Stx produced by O157 in the presence of colicins extracted from colicinogenic strains was assayed. The Stx family comprises two main groups, Stx1 and Stx2. Each toxin is typically encoded in the genome of the lysogenic lambdaoid phage, and their characteristics differ somewhat. Consequently, 10 O157 strains producing Stx1 and/or Stx2 (or Stx2vha) were used in the experiments. In six strains of Stx2-producing O157 and three strains possessing the Stx2 variant gene, DNase colicins E8/9 clearly enhanced Stx2 or Stx2vha production (2- to 32-fold), irrespective of the presence of Stx1, compared to results for controls cultured without colicins (Fig. 2); however, RNase colicins E3/5 showed little effect except for inhibition in two strains (strains 0001 and 20517). In contrast, no colicins clearly enhanced Stx1 production in any of the four strains, and not even mitomycin C increased Stx1 production in strain no. 0001 or the mutant of strain no. 96-98-83 (data not shown).

Growth of O157 was inhibited depending on the colicin concentration until 2 h of culture; subsequently, CFU of O157 gradually increased and reached near-stationary phase at 8 h (Fig. 3A). Stx2 production was increased by addition of more than 400 AU colicin E9 (Fig. 3B).

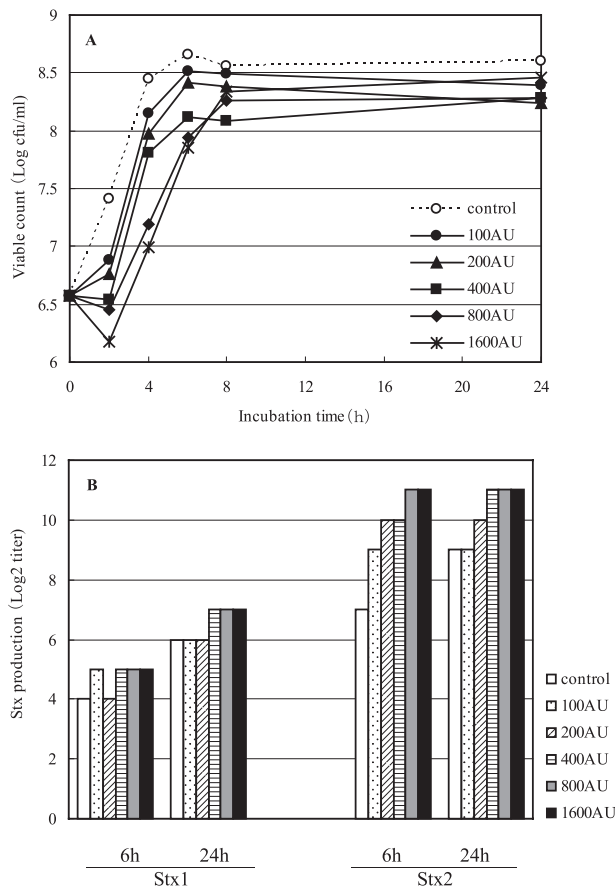


FIG. 3. Dose-dependent induction of Stx production by colicin E9. *Escherichia coli* O157 strain 96-98-83 was cultured in the presence of colicin E9, and viable counts (A) and concentrations of Stx (B) were examined. The reciprocal of the highest twofold serial dilution causing latex agglutination was recorded as the titer and is expressed as a binary logarithmic value. Signals: viable count of *E. coli* O157 after addition of colicin E9 at 100 AU, 200 AU, 400 AU, 800 AU, or 1,600 AU. The control was cultured without colicin. Representative plots of two dose-response experiments are shown.

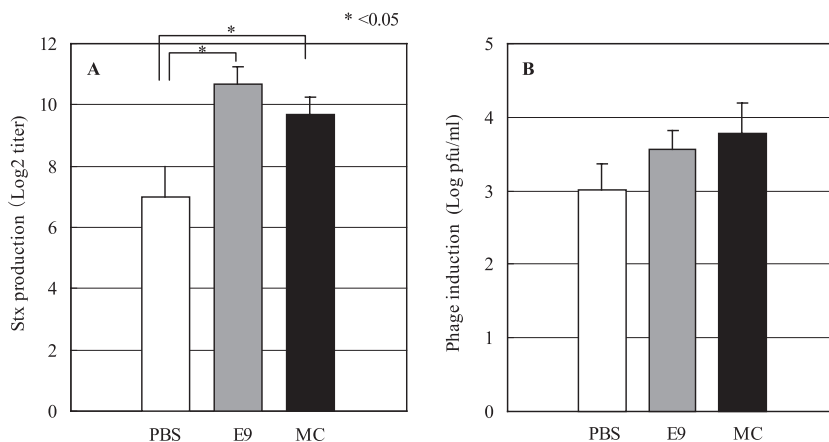


FIG. 4. Effects of colicins on Stx production and induction of prophages. *Escherichia coli* O157 strain 3001 was cultured in the presence of colicin E9 for 6 h, and Stx production (A) and numbers of phages induced in supernatants (B) were examined. The reciprocal of the highest twofold serial dilution causing latex agglutination was recorded as the titer and is expressed as a binary logarithmic value. The number of phages was determined by plaque assay. Columns show PBS (control), addition of colicin E9 at 800 AU (E9), and mitomycin C (MC) (positive control, 25 ng/ml). Data (means \pm standard errors) were from three independent experiments.

Mechanism of DNase colicin-induced Stx₂ production. It is known that the genes encoding Stx are encoded in the genome of heterogeneous lambdoid prophages (Stx phages) in O157 and that induction of Stx phages triggers increased production of Stx. We investigated the amount of Stx and the number of prophages induced by O157 in the presence of colicin, and the stimulatory effects of DNase colicin E9 (800 AU) were compared with those of mitomycin C. Colicin increased Stx₂ production to a degree comparable to that with mitomycin C at 25 ng/ml; the amount of Stx₂ produced in the presence of colicin or mitomycin C was significantly higher than that with the controls ($P < 0.05$) (Fig. 4A). Although colicin and mitomycin C induced phages to a greater degree than in controls at 6 h, the differences were not significant ($P < 0.10$) (Fig. 4B). Plaque PCR confirmed that the phages were Stx₂-converting bacteriophages.

The induction of the SOS response causes high-level expression of previously silent prophage genes, and any links between the SOS response and prophage induction for Stx gene expression are potentially important. To clarify the effects of colicin on expression of SOS response-associated genes and Stx₂, the respective mRNAs were amplified by RT-PCR. Although expression of the *recA* gene was not observed in controls, the band was amplified in the RNA samples extracted from O157 cultured with colicin (Fig. 5). Semiquantitative real-time RT-PCR was then used as a more sensitive and quantitative method. In addition to 25 ng/ml mitomycin C (positive control), DNase colicin E9 clearly enhanced the expression of SOS-related and Stx₂ genes. Mean relative gene expression values were 1.6- to 13.8-fold higher than those for controls (Fig. 6), but significant differences were not observed between the colicin-treated and mitomycin C-treated groups.

DISCUSSION

Microbiota are expected to play an important role in the prevention and/or amelioration of intestinal infections (12). Our previous studies have suggested that people possessing

colicinogenic bacteria that inhibit O157 tend not to manifest hemorrhagic colitis (28). We hypothesized that colicinogenic bacteria play a role in preventing the deleterious effects of O157. However, coculture with RNase-colicin-producing strains had no effect on production, and RNase colicins suppressed Stx production in only two strains. The present findings indicate that DNase colicins (colicins E8 and E9) enhance production of Stx₂ by O157. Both extracted colicins and the DNase colicin-producing strains themselves induced Stx₂ production in culture with O157. To our knowledge, this is the first report showing the stimulatory effects of colicins on Stx production, although we originally expected colicins to exert inhibitory effects on Stx production.

DNase colicins induced prophages and increased Stx₂ production. Quinolones, which are inhibitors of bacterial DNA

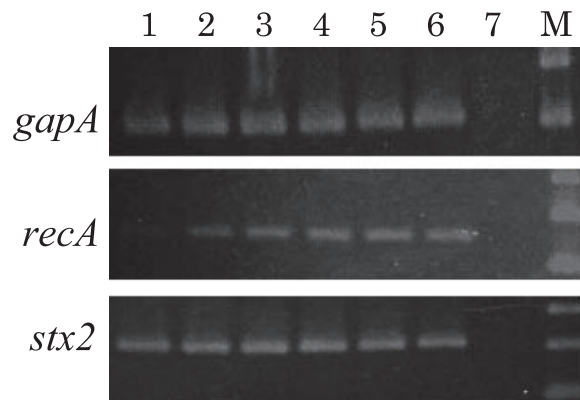


FIG. 5. Effects of colicins on expression of SOS response-associated genes. *Escherichia coli* O157 strain 3001 was cultured in the presence of 800 AU of colicin E9 (lane 2) or 50, 25, or 12.5 ng/ml mitomycin C (lanes 3, 4, and 5, respectively). Lane 1 is the control. After 6 h of incubation at 37°C, RNA was extracted and used as a template (lanes 1 to 5) for RT-PCR. DNAs of *E. coli* O157 strain 3001 (lane 6) and D. W. (lane 7) were used as positive and negative control templates.

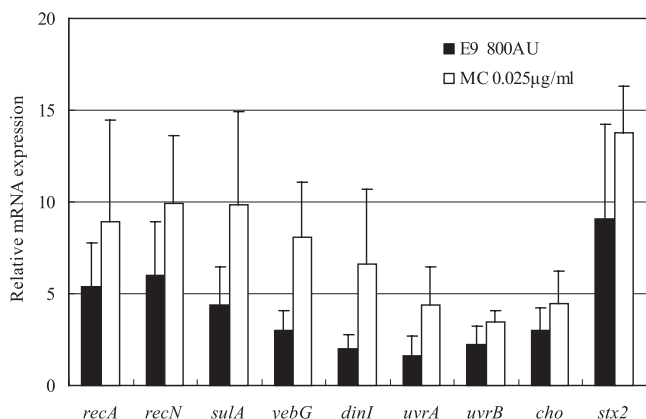


FIG. 6. Effects of colicins on transcription of SOS reaction-associated genes. *Escherichia coli* O157 strain 3001 was cultured in the presence of 800 AU of colicin E9, mitomycin C, or PBS (control). After 6 h of incubation at 37°C, RNA was extracted. Real-time RT-PCR assays were performed using SYBR green on a PRISM 7000 sequence detection system. Data are expressed as multiples relative to the control value. Columns show addition of colicin E9 at 800 AU or mitomycin C. Data (means \pm standard errors) are from three independent experiments. Significant differences between data from colicin- and mitomycin-treated groups were evaluated for each gene using Student's unpaired *t* test or Welch's *t* test after an F test was performed.

gyrase, accelerate Stx production in O157 (8–10, 13, 32). Mitomycin C, which damages DNA by cross-linking complementary strands, and irradiation also reportedly increased Stx production (31). Walker et al. reported that the DNase colicin E9 induced 28 genes of the LexA-regulated SOS response in *E. coli* K12 strain MG1655 (30). In combination with these reports, our findings strongly suggest that the DNA damage caused by the DNase colicins activates the SOS system, resulting in cleavage of the CI phage repressor and enhanced Stx production. In fact, RT-PCR and semiquantitative real-time RT-PCR revealed obvious increases in the expression of SOS-associated genes (23) and the Stx2 gene (Fig. 6), although the stimulatory effects of DNase colicins (800 AU) are modest, being equivalent to 25 ng/ml mitomycin C.

Compared to Stx2 production, production of Stx1 did not show any clear induction by DNase colicins. CI phage repressors work to regulate the switch between lysis and lysogeny by binding to operator regions. Lambdoid prophage induction requires inactivation of repressor DNA binding activity, and this inactivation can be caused by the DNA damage-induced active form of RecA. Koudelka et al. reported that the Stx2 phage (933W) was comparatively unstable, since operator region 2 has relatively low affinity for the repressor (11). Patients infected with EHEC strains producing Stx2 alone or in combination with Stx1 are more likely to develop serious renal or circulatory complications than patients infected with strains producing Stx1 alone (21, 26). Previously we found that Stx2 is more likely to be associated with hemorrhagic diarrhea than Stx2 variants (19). Since the unstable nature of the Stx2 phage due to the low affinity for phage repressor results in easy phage induction and Stx2 production, Stx2-producing O157 strains are more virulent than EHEC strains possessing only Stx1.

Recently bacteriocins and producer strains have attracted

attention, since bacterial proteins can be utilized as additives for biopreservation; nisin, a bacteriocin found in lactic acid bacteria, is now commercially available as a food additive. Indeed, an E2-type DNase colicin was reported to be effective in protecting alfalfa seeds from O157 (16). Colicins are thought to be digested before arriving at the intestine, and even if they enhance Stx production in food-borne O157 organisms, Stx is unlikely to be the cause of intradietetic intoxication. However, if DNase colicins are used as food additives for biopreservation, we should carefully assess the risks, since the concentrations may be beyond physiological levels.

Interaction between resident bacteria and enteric pathogens presumably plays a critical role in pathogenesis and individual sensitivity to pathogens. Gamage et al. reported that Stx levels in mouse intestines infected with O157 increased in the presence of commensal bacteria that were susceptible to the Stx phage while phage-resistant bacteria inhibited Stx production (2). Although DNase colicin-producing bacteria slightly enhanced Stx production in cocultured O157 in this study, we expect beneficial effects to be exerted by colicinogenic bacteria as part of the microbiota unless large amounts of purified colicins are used. Stimulation of Stx production occurred only when the concentration ratio of producer to O157 was 1:1. If small numbers of ingested O157 bacteria settle in intestinal epithelia in the presence of large amounts of resident colicinogenic bacteria, such inhibitory bacteria would be beneficial irrespective of their colicin type. Furthermore, the decreased Stx production due to RNase colicins in two strains suggests that colicinogenic bacteria may contribute differently to host protection based on target O157 strains.

In conclusion, the present study indicates that DNase colicins and colicinogenic bacteria can be stimulatory factors for Stx production in O157. Further studies should be performed in order to determine the risks and benefits of using colicinogenic bacteria and colicins for bacterial interference, although a variety of studies have suggested their benefits. Adhesion of O157 organisms to intestinal epithelial cells is a prerequisite step in establishing infection (6). Intimate adherence allows O157 to efficiently transfer Stx to epithelial cells. Colicins may inhibit the expression of colonization factors, such as the type three secretion system and effectors, and even DNase-colicin-producing bacteria could thus act beneficially, nullifying the disadvantageous effects of increased Stx production. Further investigations are in progress to elucidate the effects of colicins on Stx production in vivo and on expression of the type three secretion system.

ACKNOWLEDGMENT

This study was funded by a Grant-in-Aid for Young Scientists B (no. 19700588) from the Japan Society for the Promotion of Science.

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