Journal of Food Protection, Vol. 65, No. 9, 2002, Pages 1381–1387

Selection of Recently Isolated Colicinogenic *Escherichia coli* Strains Inhibitory to *Escherichia coli* O157:H7

GERRY P. SCHAMBERGER AND FRANCISCO DIEZ-GONZALEZ*

Department of Food Science and Nutrition, University of Minnesota, 1334 Eckles Avenue, St. Paul, Minnesota 55108, USA

MS 02-42: Received 11 February 2002/Accepted 14 April 2002

ABSTRACT

Escherichia coli strains were screened for their ability to inhibit *E. coli* O157:H7. An initial evaluation of 18 strains carrying previously characterized colicins determined that only colicin E7 inhibited all of the *E. coli* O157:H7 strains tested. A total of 540 strains that had recently been isolated from humans and nine different animal species (cats, cattle, chickens, deer, dogs, ducks, horses, pigs, and sheep) were tested by a flip-plating technique. Approximately 38% of these strains were found to inhibit noncolicinogenic *E. coli* K12 strains. The percentage of potentially colicinogenic *E. coli* per animal species ranged from 14% for horse isolates to 64% for sheep strains. Those isolates that inhibited *E. coli* K12 were screened against *E. coli* O157:H7, and 42 strains were found to be capable of inhibiting all 22 pathogenic strains tested. None of these 42 strains produced bacteriophages, and only 24 isolates inhibited serotype O157:H7 in liquid culture. The inhibitory activity of these strains was completely eliminated by treatment with proteinase K. When mixtures of these 24 colicinogenic strains were grown in anaerobic continuous culture, the four-strain *E. coli* O157:H7 population was reduced at a rate of 0.25 $\log_{10}$ cells per ml per h, which was fivefold faster than the washout rate. Two strains originally isolated from cat feces (F16) and human feces (H30) were identified by repetitive sequences polymerase chain reaction as the predominant isolates in continuous cultures. The results of this work indicate that animal species other than cattle can be sources of anti-O157 colicinogenic strains, and these results also lead to the identification of at least two isolates that could potentially be used in preharvest control strategies.

Escherichia coli O157:H7 is a highly virulent foodborne pathogen that causes approximately 73,000 infections and 60 deaths every year in the United States *(15). E. coli* O157:H7 infection can be acquired through the consumption of contaminated meats, water, milk, apple cider, and raw vegetables *(5).* In a large number of outbreaks, the source of *E. coli* O157:H7 has been confirmed to be cattle that asymptomatically carry and shed the pathogen in their feces *(2).* It is now well established that bovine animals are the primary reservoir of *E. coli* O157:H7 *(8).* A significant reduction in the overall incidence of enterohemorragic colitis will stem from strategies aimed at reducing the fecal shedding of *E. coli* O157:H7 by cattle.

One proposed method for the preharvest control of *E. coli* O157:H7 is competitive exclusion, which is defined as the use of probiotic bacteria to prevent the colonization of pathogenic organisms. The reduction of *E. coli* O157:H7 can be accomplished with other nonpathogenic *E. coli* strains that produce colicins. Colicins are antimicrobial proteins produced by *E. coli* that inhibit other *E. coli* strains and closely related bacteria *(20).* The ability to produce colicins is widespread in the *Enterobacteriacea* family, and surveys have reported that approximately 30% of *E. coli* isolates can produce a colicin *(19, 22).* The type of colicin produced appears to be influenced by the species of the animal source, and resistance to noncattle colicinogenic *E. coli* would be expected to be rare in strains naturally present in cattle *(23).* Zhao et al. *(24)* developed the first competitive-exclusion system against *E. coli* O157:H7 by isolating colicinogenic *E. coli* strains from cattle, but little information was provided about the types of colicins produced by these strains and the extent of resistance among naturally present *E. coli* O157:H7 strains.

The present project was undertaken to identify colicinogenic *E. coli* strains that strongly inhibit serotype O157:H7 and could potentially be used in a competitive-exclusion strategy. Additionally, this research determined the overall distribution of colicinogeny and inhibitory activity against *E. coli* O157:H7 in isolates from humans and nine animal species.

MATERIALS AND METHODS

Strains and media. *E. coli* K12 strains CSH50 and W3110 were obtained from the *E. coli* Genetic Stock Center at Yale University. Twenty-two strains of *E. coli* O157:H7 were used: ATCC 43890, ATCC 43895, ATCC 35150, 3081, 86-24, 6058, 2026, 2027, 2028, 2029, 2030, 2031, 2079, 2255, 2257, 2266, 2309, 2317, 2321, 2324, 2336, and MAC. Seventeen of these strains were provided by Todd R. Callaway (U.S. Department of Agriculture, Agricultural Research Service, College Station, Tex.). Eighteen *E. coli* K12 strains carrying plasmids encoding previously characterized colicins (Pugsley *(18)* and data not shown) were obtained from David Gordon (Australian National University, Canberra, Australia). A total of 540 *E. coli* strains that had recently been isolated from humans and nine different animal sources *(6)* were obtained from Michael Sadowsky (University of

* Author for correspondence. Tel: 612-624-9756; Fax: 612-625-5272; E-mail: fdiez@umn.edu.

Minnesota, St. Paul, Minn.). Thirty-eight noncolicinogenic *E. coli* strains were isolated from cattle *(12)*.

Stock cultures of all strains were stored at −50°C in vials containing 50% glycerol. Strains were cultivated at 37°C in Luria-Bertani (LB) broth and in LA agar (LB broth containing 1.5% agar). For overlay assays, LA soft agar (0.75% agar) was used. LA agar plates containing mitomycin C (LAm) were prepared with the addition of 0.25 mg of mitomycin C (ICN Biomedicals Inc., Aurora, Ohio) per liter after autoclaving. The composition of the media used for continuous cultures included (per liter): 0.5 g of yeast extract, 1.0 g of Trypticase, 2.0 g of maltose, 0.6 g of cysteine hydrochloride, 5.7 g of Na_2HPO_4, 0.01 mg of resazurin, 12.0 g of KH_2PO_4, 12.0 g of $(NH_4)_2SO_4$, 12.0 g of NaCl, 2.5 g of $MgSO_4 \cdot 7H_2O$, and 1.6 g $CaCl_2 \cdot 2H_2O$. After autoclaving, purging with O_2-free nitrogen rendered the medium anaerobic. Chromagar O157 (Chromagar Microbiology, Paris, France) medium containing 1.5 mg of potassium tellurite per liter was used for the detection of *E. coli* O157:H7.

Colicin detection methods. LAm plates were spot inoculated with 25 μl of overnight LB cultures of *E. coli* and incubated at 37°C. After overnight growth, the agar layers were flipped over, resulting in the production of colonies on the bottoms of the petri plates. Plates were overlaid with 5 ml of LA soft agar containing 10^6 cells of the indicator strain and were incubated at 37°C overnight. These plates were then observed and scored against the indicator lawn for colicin production. The method used for the detection of colicin production in liquid medium was a modification of the method described by Gordon et al. *(10)*. Five-milliliter tubes containing LB broth were inoculated with 0.5 ml of overnight culture. Tubes were shaken for 1 h in an orbital shaker (200 rpm, 37°C), and 0.2 μg of mitomycin C per ml was added. Cultures were shaken for an additional 4 h and centrifuged in a microcentrifuge for 5 min at 20,000 × *g*. Supernatant aliquots (0.9 ml) were mixed with chloroform (0.1 ml), shaken for 15 min, and centrifuged for 10 min at 20,000 × *g*. The top layer of the mixture was transferred to a new tube, and the chloroform treatment was repeated. Supernatants were removed and stored at 4°C.

Supernatants were tested by direct spotting onto petri plates or by the serial-dilution technique. For the direct-spotting method, 10 μl of supernatant was spotted onto LA plates that had previously been overlaid with 5 ml of LA soft agar containing 10^6 indicator cells. The plates were incubated for 24 h at 37°C, and zones of inhibition surrounding the spotted area were measured. For the serial dilution technique, indicator cultures that had grown overnight were serially diluted 10-fold in 96-well microtiter plates containing 180 μl of LB broth and were then mixed with 20 μl of chloroform-treated supernatants. The microtiter plates were incubated overnight at 37°C, and the presence or absence of growth in the wells was observed and recorded.

Assays for proteinaceous substances and bacteriophages. To test whether the inhibitory substance was a protein, chloroform-treated supernatants were mixed with 200 μg of proteinase K (Sigma Chemical Co., St. Louis, Mo.) per ml, shaken for 30 min, and spotted as described above. An untreated supernatant was also spotted onto indicator plates as a control. The plates were incubated for 24 h at 37°C, and the lack of a zone of inhibition in a treated-supernatant plate was considered evidence of the presence of colicins. To test whether the inhibitory substance was a bacteriophage, a procedure was adapted from Pugsley and Oudega *(20)*. Circular pieces of agar were cut from zones of lysis on LA plates and resuspended in 1 ml of LB broth and 50 μl of chloroform in a microcentrifuge tube. Tubes were vortexed and incubated for 5 min, and 100-μl aliquots were transferred into 5-ml LA soft agar tubes containing 10^6 indicator cells, poured onto LA agar plates, and incubated overnight at 37°C. Typical plaque formation would indicate the presence of bacteriophages.

Continuous-culture experiments. The chemostat experiments were conducted with Omni-Culture Bench-Top Fermenters (500-ml working volume; Virtis Co., Inc., Gardiner, N.Y.) that were operated at 220 rpm at 37°C. The pH was kept at 6.8 ± 0.1 with autoclavable pH electrodes and the automatic addition of 3 M HCl or 3 M NaOH. The continuous-culture vessels containing fresh CC media were inoculated with a mixture of *E. coli* strains (either colicinogenic or noncolicinogenic) containing 1 ml of overnight cultures of each strain. The strain mixtures were allowed to grow for 5 h, and fresh medium was then pumped in at a dilution rate of 0.1 h^{-1}. Daily samples were collected from chemostats to determine pH, optical density at 600 nm, total *E. coli* counts, and *E. coli* O157:H7 counts. Four *E. coli* O157:H7 strains were introduced into the chemostats approximately 65 h after the initial inoculation to obtain a 99.9% turnover rate from the initial population. A 1% *E. coli* O157:H7 inoculum (approximately 10^6 cells per ml) consisting of 1.25 ml of overnight LB cultures of each *E. coli* O157:H7 strain (3081, ATCC 35150, ATCC 43890, ATCC 43895) was transferred into the chemostat.

Microbiological analysis. Total *E. coli* counts in the chemostats were determined by the three-tube most-probable-number (MPN) technique after serial dilution from 10^{-1} to 10^{-11} into tubes containing LB broth, followed by overnight incubation at 37°C. Turbid tubes were recorded as positive for growth, and a total MPN population was determined. Samples of cultures of the most diluted tubes were transferred into vials containing 50% glycerol, mixed, and stored at −50°C for the further identification of predominant colicinogenic strains. The presence of *E. coli* O157:H7 in the MPN tubes was determined by transferring 50 μl onto Chromagar O157 petri plates and incubating these plates overnight at 37°C. The presence of pink-mauve colonies was taken to indicate the presence of *E. coli* O157:H7, and the original dilution of the MPN tubes was used to estimate the count of *E. coli* O157:H7. The ability of this method to recover as little as one *E. coli* O157:H7 cell was determined from a standard curve of mixtures of different *E. coli* O157:H7 dilutions with chemostat cultures.

Identification of predominant colicinogenic isolates. Fingerprinting of chemostat isolates was achieved by the repetitive sequences polymerase chain reaction (rep-PCR) technique described by Dombek et al. *(6)*. Stored cultures from MPN determinations were transferred into LA agar and grown overnight at 37°C. Cell templates were made by mixing colonies with 100 μl of sterile water. Two microliters of cell templates was mixed with 23 μl of PCR reaction mixture containing 45 pmol of BOX A1R primer (5′-CTACGGCAAGGCGACGCTGACG-3′), 1.25 mM each deoxynucleoside triphosphate (Fisher Scientific), 1 U of *Taq* DNA polymerase (Fisher Scientific), 5 μl of Gitschier buffer *(13)*, 2.5 μl of dimethyl sulfoxide, and 160 μg of bovine serum albumin per ml. The PCR reaction was performed with a Stratagene Robocycler model Gradient 96 at 95°C for 2 min, followed by 30 cycles of 93°C for 30 s, 50°C for 1 min, and 65°C for 8 min, followed by a final extension at 65°C for 8 min, and then the samples were held at 4°C until electrophoresis was performed. Fourteen microliters of the PCR reaction was mixed with 8 μl of water and 2 μl of loading dye. The samples were electrophoresed on a 1.5% agarose gel at 4°C for 18 h at 60 V. A 1-kb DNA fragment ladder was used as a standard. The gels were stained with a 0.5-μg/ml ethidium bromide solution for 20 min. Gel pic-

TABLE 1. *Sensitivity of* E. coli *K12 and O157:H7 strains to colicinogenic* E. coli *K12 strains carrying previously characterized colicins*[a]

Colicin type of producing strains	Inhibition of *E. coli* O157:H7 strain						
	43890	43895	3081	6058	2026	2031	2321
A	−	−	−	−	−	−	−
B	+/−	−	−	−	−	−	−
D	++	−	−	−	−	−	−
E1	+/−	−	−	−	−	−	−
E2	−	−	−	−	−	−	−
E3	++	−	−	+	−	+	−
E4	+	−	−	−	−	−	−
E5	+	−	−	−	−	−	−
E6	+++	−	−	−	−	−	+/−
E7	+++	++	++	++	++	++	+++
E8	+++	++/−	+	−	−	−	−
E9	+	−	−	−	−	−	−
Ia	++	+/−	−	+	−	+	+/−
Ib	+	+/−	−	+/−	−	−	+/−
K	++/−	+/−	−	+/−	−	−	+/−
M	−	−	−	−	−	−	−
N	++/−	−	−	−	−	−	−
V	−	−	−	++/−	+++/−	+++/−	+/−

[a] Observations were collected from two independent experiments. +++, clear inhibition zones more than 10 mm in diameter; ++, clear inhibition zones 5 to 10 mm in diameter; +, clear inhibition zones less than 5 mm in diameter or hazy zones less than 10 mm in diameter; −, no inhibition zones; ++/− or +/−, one of the replicates showed no inhibition.

tures were obtained, and band patterns of individual isolates were compared with a Gel-Doc 8000 gel documentation system (UVP, Inc.).

Calculations and data analyses. The *E. coli* O157:H7 washout rate was calculated by estimating the bacterial count on the basis of the chemostat theory *(7)* assuming that no growth occurred. For this calculation, we used the following equation:

$$\ln(X_2/X_1) = -\mu t \quad (1)$$

where X_1 is the initial bacterial count at time 0 (expressed in natural numbers), X_2 is the count after time t, and μ is the dilution rate (0.1 h^{-1}). We solved

$$X_2 = X_1 e^{-\mu t} \quad (2)$$

and plotted bacterial count versus time. The washout rate was calculated from the graph slope. Differences between *E. coli* O157:H7 cell count means in chemostat experiments were determined with Student's t test *(16)*. Significant colicinogeny differences were determined by calculating the odds ratio according to Bland and Altman *(3)*.

RESULTS

With the flip-plating procedure, a total of 17 colicinogenic *Escherichia coli* strains carrying plasmids for previously characterized colicins were able to inhibit both K12 indicator strains (data not shown). Strain PAP1 (colicin M) had little effect on the indicators. When the method involving the supernatants of induced cultures was tested, only 5 colicinogenic strains were found to have wide zones of inhibition comparable to those found with the flip-plating technique; a total of 11 strains showed some level of inhibition, and 7 strains did not produce any clearing zone. The flip-plating technique was then selected for further screening of colicinogenic *E. coli* strains.

When the colicinogenic *E. coli* K12 strains were tested against seven *E. coli* O157:H7 strains, most of the pathogenic strains were found to be resistant to colicinogenic colonies (Table 1). Among the O157:H7 strains, only ATCC 43890 was affected by as many as 14 colicinogenic strains; the rest were inhibited by less than 7 colicinogenic strains. The colicin E7–producing strain BZB2110 was the only colicinogenic K12 strain that inhibited all 7 *E. coli* O157:H7 strains, and further testing showed that BZB2110 inhibited all 22 of the O157:H7 strains studied.

A total of 540 *E. coli* strains that had recently been isolated from humans and nine different animal species (cats, cattle, chickens, deer, dogs, ducks, horses, pigs, and sheep) were screened for their ability to inhibit 2 noncolicinogenic *E. coli* K12 strains and 22 *E. coli* O157:H7 strains (Table 2). Approximately 38% of all strains inhibited both W3110 and CSH50, and the percentage of inhibitory *E. coli* per animal species ranged from 14% for horse isolates to 64% for sheep strains. Forty-two strains were found to be capable of inhibiting all 22 *E. coli* O157:H7 strains tested. The percentage of strains that inhibited *E. coli* O157:H7 was 8%, but this percentage ranged from 1% for chickens to 25% for cats. The percentage of anti-O157:H7 isolates based on the number of strains that inhibited K12 was only 5% for chickens, but it was as high as 50% for cats. Only 14% of the colicinogenic strains isolated from cattle were found to inhibit serotype O157:H7.

None of the 42 strains capable of inhibiting all 22 *E. coli* O157:H7 strains tested positive for bacteriophages when agar plugs were taken from the zones of inhibition of each potentially colicinogenic strain and visually screened for their ability to form characteristic plaques. When tested for colicin production in liquid LB broth, the

TABLE 2. *Distribution of* E. coli *strains among sources and the inhibitory activity of these strains against* E. coli *K12 and O157:H7 strains*[a]

Source	No. of isolates	No. (%) of isolates inhibitory to K12 (strains W3110 and CSH50)	No. (%) of isolates inhibitory to 22 O157:H7 strains	% of isolates inhibitory to O157:H7 based on no. inhibitory to K12
Cats	32	16 (50)	8 (25)[b]	50
Cattle	77	35 (45)	5 (6)	14
Chickens	67	21 (31)	1 (1)	5
Deer	43	27 (63)[b]	2 (5)	7
Dogs	46	12 (26)	4 (9)	33
Ducks	40	12 (30)	2 (5)	17
Horses	36	5 (14)[b]	1 (3)	20
Humans	121	35 (29)	10 (8)	29
Pigs	42	18 (43)	2 (5)	11
Sheep	36	23 (64)[b]	7 (19)[b]	30
Total	540	204 (38)	42 (8)	21

[a] Only one isolate was obtained from the feces of a single individual or animal. Values are combined scores for individual observations for 2 different K12 strains and 22 different O157:H7 strains.
[b] Statistically significant on the basis of the odds ratio at a 95% confidence level.

supernatants of 18 strains were not found to inhibit liquid cultures of strain 43890 containing as little as 1 cell per ml, even after induction with mitomycin C. The inhibitory activity of the supernatants of the remaining 24 strains was completely eliminated by incubation with proteinase K. Fourteen colicinogenic strains killed at least 10^6 cells of strain 43890 per ml (Table 3). When the colicinogenic cultures were not induced with mitomycin C, the inhibition of 43890 was markedly decreased, and only five strains were found to inhibit 10^3 cells per ml.

Mixtures of the 24 colicinogenic strains growing in continuous cultures had an average optical density at 600 nm of approximately 0.6 (Fig. 1a) and a total viable count of 10^9 cells per ml (Fig. 1b). The optical density and the total viable count were not significantly affected by the addition of four *E. coli* O157:H7 strains to the chemostats. The *E. coli* O157:H7 population was markedly reduced (from 10^7 to 10^3 cells per ml) during the first 24 h and was almost undetectable after 90 h. However, the *E. coli* O157:H7 count slightly increased to 100 cells per ml after 180 h and remained at that level or lower throughout the remainder of the experiments. The supernatant of continuous cultures, however, was not found to inhibit *E. coli* O157:H7 when assayed on liquid or petri plate cultures.

When similar continuous-culture experiments were conducted with mixtures of noncolicinogenic *E. coli* strains isolated from cattle, the optical density was approximately 0.7 (Fig. 2a), and the total *E. coli* count was typically 10^9

TABLE 3. *Effect of addition of mitomycin C to cultures of 24 selected colicinogenic* E. coli *strains on the maximum number of* E. coli *O157:H7 ATCC 43890 cells killed by culture supernatants for each animal source*

Source	No. of isolates	No. of cells killed with uninduced cultures ($\log_{10}$ cells/ml ± SD)	No. of cells killed with mitomycin C–induced cultures ($\log_{10}$ cells/ml ± SD)
Cats	3	1.7 ± 1.2	6.3 ± 0.6
Cattle	3	2.3 ± 0.6	6.3 ± 0.6
Deer	2	1.0 ± 0.0	5.0 ± 0.0
Ducks	1	2.0 ± 0.0	7.0 ± 0.0
Humans	6	1.8 ± 1.6	5.7 ± 1.5
Pigs	2	1.0 ± 0.0	4.5 ± 0.7
Sheep	7	1.2 ± 1.2	6.7 ± 1.0

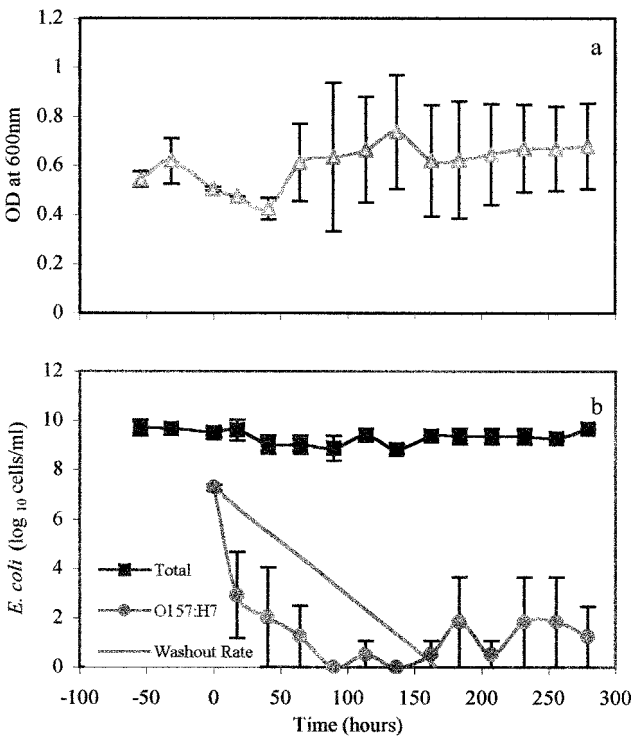

FIGURE 1. *Ability of* E. coli *O157:H7 to compete against a population of 24 colicinogenic* E. coli *strains isolated from humans and six animal species in continuous culture at pH 6.8. (a) Optical density. (b)* E. coli *count. Four O157:H7 strains were added at time 0. The dilution rate was 0.1 h^{-1}. Data points are averages of two independent experiments (CC1 and CC2).*

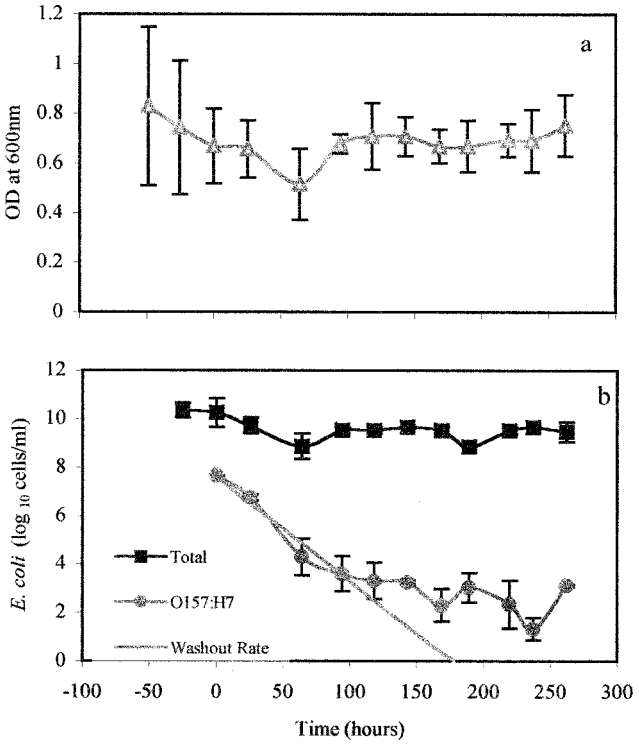

FIGURE 2. *Ability of* E. coli *O157:H7 to compete against a population of 38 noncolicinogenic* E. coli *strains isolated from cattle in continuous culture at pH 6.8. (a) Optical density. (b)* E. coli *count. Four O157:H7 strains were added at time 0. The dilution rate was 0.1 h^{-1}. Data points are averages of two independent experiments.*

cells per ml throughout the experiments (Fig 2b). After the addition of the mixture of pathogenic strains, the *E. coli* O157:H7 population gradually decreased to approximately 10^4 cells per ml after 100 h and remained at an average of 10^3 cells per ml during the last stages of the cultures.

Rep-PCR analysis of the 24 colicinogenic *E. coli* strains revealed 23 visually distinct band patterns, and only strains P37 and P39 were indistinguishable from each other (data not shown). Predominant colicinogenic *E. coli* isolates obtained from chemostats were subjected to rep-PCR, and only strains F16 and H30 were identified. In one of the chemostat trials (CC2), only strain F16 was always predominant after reaching steady state, but in a replicate experiment (CC1), F16 was predominant during the first 110 h and at 180 h. Strain H30 was predominant in chemostat CC1 at 136 h, at 163 h, and after 203 h. The population of *E. coli* O157:H7 in chemostat CC2 was consistently smaller than that in chemostat CC1 (data not shown).

DISCUSSION

According to Riley *(21),* as many as 25 different types of colicins have been classified, and Pugsley *(18)* originally developed a culture collection of K12 derivatives with 18 different colicins. This collection has been available for typing and reference, and it has recently been used to identify colicins that inhibit *E. coli* O157:H7 *(4, 17).* From the Pugsley collection, 18 standard colicinogenic *E. coli* K12 strains (each producing a unique colicin) were screened against 7 strains of *E. coli* O157:H7. When the colicinogenic *E. coli* K12 strains were tested against 7 *E. coli* O157:H7 strains by the flip-plating assay, most of the pathogenic strains were found to be resistant to the colicins. The colicin E7–producing strain was the only colicinogenic K12 strain that was found to inhibit as many as 22 O157:H7 strains. These results clearly indicate that this standard colicinogenic collection is not a viable source of anti-O157 strains and colicins.

Despite the observation that only one colicin failed to inhibit both strain CSH50 and strain W3110, it was surprising to find that previously characterized colicinogenic strains had little inhibitory activity against O157:H7 strains. In addition, this lack of inhibition conflicts with previously published reports *(4, 17).* Bradley et al. *(4)* reported that colicin E2 and colicin V inhibited 12 and 18 O157:H7 strains, respectively, but in the present study, colicin E2 was not found to inhibit any strains and colicin V was found to inhibit 4 of 7 strains of *E. coli* O157:H7. Murinda et al. (17) reported the ability of 13 colicins to inhibit 11 *E. coli* O157:H7 strains. In spite of the similarity between the assay techniques used, it is likely that these contradictory results might be due to the different *E. coli* O157:H7 strains used among the three research groups, as these strains may possess diverse sensitivities to colicins.

To identify additional colicinogenic strains capable of inhibiting *E. coli* O157:H7, a total of 540 *E. coli* strains recently isolated from humans and nine different animal species (cats, cattle, chickens, deer, dogs, ducks, horses, pigs, and sheep) were first screened for their ability to inhibit two noncolicinogenic *E. coli* K12 strains. This variety of animal species was chosen because *E. coli* O157:H7 is naturally present in cattle, and the type of colicin produced by *E. coli* strains present in the intestine appears to be influenced by the species of the animal *(23).* According to Riley and Gordon *(23),* colicins M and V were found more frequently in chickens than in humans, and the *E. coli* naturally present in poultry would likely be more resistant to these colicins. Since *E. coli* O157:H7 is strongly associated with cattle, we hypothesized that the pathogen would more frequently be resistant to colicins produced by *E. coli* strains isolated from cattle. By targeting *E. coli* isolates from other animal species, we could potentially avoid specific colicin resistance already present in *E. coli* O157:H7 strains.

The overall percentage (38%) of the 540 screened *E. coli* strains that inhibited K12 strains was very close to colicinogeny estimates of 30 and 35% previously reported by Pugsley *(19)* and Riley and Gordon *(22),* respectively. When the percentage was calculated for each animal species, however, a wide range of colicinogeny was found. Horse isolates had the lowest prevalence of inhibitory *E. coli* (14%), but up to 63% of isolates from sheep and deer exhibited potentially colicinogenic activity. Similar colicinogeny variability was noted by Riley and Gordon *(22),* who screened a human-animal *E. coli* reference collection and found that 50% of human strains were colicinogenic, while only 16% of animal isolates appeared to produce colicin. These authors suggested that colicinogeny was more

widespread in human isolates than in animal isolates *(23)*. In contrast, approximately 29% of the human isolates appeared to be colicinogenic, while 40% of all of the animal strains were found to be inhibitory.

In the next selection stage, the overall percentage of *E. coli* strains that were inhibitory to all *E. coli* O157:H7 strains was 8%, and the percentage of colicinogenic strains that were inhibitory to all *E. coli* O157:H7 strains was 21%. Colicinogeny against serotype O157:H7 was also variable, but the percentage of potentially colicinogenic strains was between 3 and 9% for most animal species. When the number of strains inhibitory to O157:H7 was calculated as the percentage of strains that inhibited the K12 strains, isolates from cats had the highest anti-O157 prevalence. Colicinogenic cattle isolates that inhibited serotype O157:H7 accounted for 14% of the potentially colicinogenic cattle strains. Because a total of 42 strains were capable of inhibiting all 22 pathogenic strains, it appears that this group could serve as a promising source of anti-O157 strains. This is the first report investigating colicinogeny against *E. coli* O157:H7 in diverse *E. coli* populations obtained from different animal species.

According to the chemostat theory originally developed by Monod, the major factor that defines competition by different strains is the affinity constant (K_s) for the limiting substrate *(7)*. In the present study, the initial adaptation period (68 h after inoculation) selected for the noncolicinogenic *E. coli* strain or strains with the lowest K_s values. For the colicinogenic-strain mixture, the sensitivity of some strains to colicins produced by other strains was likely an additional selection factor. On the basis of these considerations (Figs. 1 and 2), it was not surprising that *E. coli* O157:H7 strains could not compete against the colicinogenic and noncolicinogenic strains that had been subjected to selection among the 24 and 38 strains, respectively. The difference in the rates of disappearance of the pathogenic strains appeared to be largely due to colicin production.

Continuous cultivation of mixed populations of colicinogenic and sensitive *E. coli* strains has previously been studied with chemostat and repeated-transfer cultivation. Adams et al. *(1)* investigated chemostat co-cultures of a colicin E1–producing *E. coli* strain and a colicin-sensitive *E. coli* K12 strain. These authors reported that the colicinogenic strain overcompeted the sensitive strain only if the latter comprised less than 50% of the total population, and they concluded that this effect was dependent on the initial strain frequency. More recently, Gordon and Riley *(9)* used a successive-transfer model with a pair of sensitive and colicinogenic strains, and they observed that the colicinogenic strain always displaced the sensitive one regardless of the initial frequency. The present study involved a mixture of colicinogenic strains and four sensitive *E. coli* O157:H7 strains growing in chemostats as a selection tool to identify potentially useful anti-O157 colicinogenic *E. coli* strains. This study did not address the effect of frequency, but to our knowledge this is the first report on the use of a complex colicinogenic strain mixture for competition studies involving continuous cultures.

In this study, a mixture of colicinogenic *E. coli* strains with high inhibitory activity was used to select those strains that not only would be able to compete with *E. coli* O157:H7 but could markedly reduce its population. When the four pathogenic strains were used to challenge chemostats inoculated with previously adapted noncolicinogenic strains, the O157:H7 serotypes could not compete, and their population in the chemostat was reduced at a rate similar to the washout rate (0.045 $\log_{10}$ cells per ml per h) until it reached 10^3 cells per ml (Fig. 2); however, when the pathogenic strains were added to chemostats containing colicinogenic strains, their populations were reduced at a five-fold-faster rate within 17 h after challenge and were almost completely eliminated from the chemostats after 90 h. This difference suggests that the O157:H7 population was killed in the colicinogenic chemostats.

According to Monod's chemostat theory, when a mixture of strains is grown in continuous culture, only one strain would be expected to remain in the culture vessel after a very long period of cultivation *(7)*. However, in the first stages, a few strains with substrate affinities relatively similar to those of the predominant strain would be expected to prevail in the chemostat. Jarvis et al. *(12)* used a chemostat system similar to the one used in this investigation to study competition among *E. coli* strains isolated from cattle and observed that as many as three strains were present in the culture vessel after 5 days of cultivation at a dilution rate of 0.1 h^{-1}. These authors also reported that it took 20 days for a single strain to become 100% predominant. In our experiments, we detected the predominant colicinogenic strain only in the MPN tubes at the highest dilution for which growth was observed, and strain F16 appeared to be the prevailing strain throughout the replicate experiments. F16 was consistently identified at $\geq 10^9$ cells per ml after the first day of cultivation. However, in the later stages of chemostat CC1, another strain (H30) became the predominant isolate, and its presence appeared to be related to a less marked reduction of *E. coli* O157:H7 strains.

The application of colicins or colicinogenic strains to the prevention and treatment of disease has been explored since the early 1900s. At least two commercial preparations (colicin X and colibacterin) were widely commercialized for the treatment of bacillary dysentery in humans *(11)*. With the emergence of gram-negative infections caused by *E. coli* O157:H7, a renewed interest in these antimicrobial proteins has driven research focused on their application to the control of this foodborne pathogen. Zhao et al. *(24)* developed the first competitive-exclusion system to reduce the fecal shedding of *E. coli* O157:H7 by cattle with a combination of 17 colicinogenic *E. coli* strains. Lyon and Olson *(14)* isolated and characterized a novel DNAase colicin (ECL 12) from swine, and this colicin was used to reduce the count of *E. coli* O157:H7 in ground beef. The colicins produced by strains F16 and H30 identified in this report will be characterized, and their potential use will be evaluated. The promising characteristics of the two colicinogenic strains suggest that they could be good candidates for a control strategy against *E. coli* O157:H7, such as a competitive-exclusion system.

ACKNOWLEDGMENTS

Funding for this project was provided by a grant-in-aid program from the University of Minnesota's Graduate School and by the Minnesota Agricultural Experiment Station. The authors thank Michael Sadowsky, David Gordon, and Todd Callaway for providing *E. coli* strains.

REFERENCES

1. Adams, J., T. Kinney, S. Thompson, L. Rubin, and R. B. Helling. 1979. Frequency-dependent selection for plasmid-containing cells of *Escherichia coli. Genetics* 91:627–637.
2. Armstrong, G. L., J. Hollingsworth, and J. Glenn Morris. 1996. Emerging foodborne pathogens: *Escherichia coli* O157:H7 as a model of entry of a new pathogen into the food supply of the developed world. *Epidemiol. Rev.* 18:29–51.
3. Bland, J. M., and D. G. Altman. 2000. The odds ratio. *Br. Med. J.* 320:1468.
4. Bradley, D. E., S. P. Howard, and H. Lior. 1990. Colicinogeny of O157:H7 enterohemorrhagic *Escherichia coli* and the shielding of colicin and phage receptors by their O-antigenic side chains. *Can. J. Microbiol.* 37:97–104.
5. Buchanan, R. L., and M. P. Doyle. 1997. Foodborne disease significance of *Escherichia coli* O157:H7 and other enterohemorrhagic *E. coli. Food Technol.* 51:69–76.
6. Dombek, P. E., L. K. Johnson, S. T. Zimmerly, and M. J. Sadowsky. 2000. Use of repetitive DNA sequences and the PCR to differentiate *Escherichia coli* isolates from human and animal sources. *Appl. Environ. Microbiol.* 66:2572–2577.
7. Dykhuizen, D. E., and D. L. Hartl. 1983. Selection in chemostats. *Microbiol. Rev.* 47:150–168.
8. Elder, R. O., U. E. Keen, G. R. Siragusa, G. A. Barkocy-Gallagher, M. Koohmaraie, and W. W. Laegreid. 2000. Correlation of enterohemorrhagic *Escherichia coli* O157 prevalence in feces, hides, and carcasses of beef cattle during processing. *Proc. Natl. Acad. Sci. USA* 97:2999–3003.
9. Gordon, D. M., and M. A. Riley. 1999. A theoretical and empirical investigation of the invasion dynamics of colicinogeny. *Microbiology* 145:655–661.
10. Gordon, D. M., M. A. Riley, and T. Pinou. 1998. Temporal changes in the frequency of colicigeny in *Escherichia coli* from house mice. *Microbiology* 144:2233–2240.
11. Hardy, K. G. 1975. Colicinogeny and related phenomena. *Bacteriol. Rev.* 39:464–515.
12. Jarvis, G. N., M. G. Kizoulis, F. Diez-Gonzalez, and J. B. Russell. 2000. The genetic diversity of predominant *Escherichia coli* strains isolated from cattle fed various amounts of hay and grain. *FEMS Microbiol. Ecol.* 32:225–233.
13. Kogan, S., M. Doherty, and J. Gitschier. 1987. An improved method for diagnosis of genetic diseases by amplified DNA sequences. *N. Engl. J. Med.* 317:985–990.
14. Lyon, W. J., and D. G. Olson. 1997. Isolation and purification of colicin ECL 12, a bacteriocin that inhibits strains of *Escherichia coli* O157:H7. *J. Food Prot.* 60:1520–1528.
15. Mead, P. S., L. Slutsker, V. Dietz, L. F. McCaig, J. S. Bresee, C. Shapiro, P. M. Griffin, and R. V. Tauxe. 1999. Food-related illness and death in the United States. *Emerg. Infect. Dis.* 5:607–625.
16. Moore, D. S., and G. P. McCabe. 1999. Introduction to the practice of statistics, 3rd ed. W. H. Freeman and Co., New York.
17. Murinda, S. E., R. F. Roberts, and R. A. Wilson. 1996. Evaluation of colicins for inhibitory activity against diarrheagenic *Escherichia coli* strains, including serotype O157:H7. *Appl. Environ. Microbiol.* 62:3196–3202.
18. Pugsley, A. P. 1984. *Escherichia coli* K12 strains for use in the identification and characterization of colicins. *J. Gen. Microbiol.* 131:369–376.
19. Pugsley, A. P. 1984. The ins and outs of colicins. Part I: production, and translocation across membranes. *Microbiol. Sci.* 7:168–175.
20. Pugsley, A. P., and B. Oudega. 1987. Methods for studying colicins and their plasmids, p. 105. *In* K. G. Hardy (ed.), Plasmids: a practical approach. IRL Press, Washington, D.C.
21. Riley, M. A. 1998. Molecular mechanisms of bacteriocin evolution. *Annu. Rev. Genet.* 32:255–278.
22. Riley, M. A., and D. M. Gordon. 1992. A survey of Col plasmids in natural isolates of *Escherichia coli* and an investigation into the stability of Col-plasmid lineages. *J. Gen. Microbiol.* 138:1345–1352.
23. Riley, M. A., and D. M. Gordon. 1996. The ecology and evolution of bacteriocins. *J. Ind. Microbiol.* 17:151–158.
24. Zhao, T., M. P. Doyle, B. G. Harmon, C. A. Brown, P. O. E. Mueller, and A. H. Parks. 1998. Reduction of carriage of enterohemorrhagic *Escherichia coli* O157:H7 in cattle by inoculation with probiotic bacteria. *J. Clin. Microbiol.* 36:641–647.