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Characterization of Colicinogenic *Escherichia coli* Strains Inhibitory to Enterohemorrhagic *Escherichia coli*

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ABSTRACT

A previously identified set of anti–*Escherichia coli* O157:H7 colicinogenic *E. coli* were characterized to assess the suitability of these isolates as a preharvest food safety intervention in cattle. This collection of 23 *E. coli* strains were screened for virulence factors, antibiotic resistance, type of colicin(s) present, and their ability to inhibit other pathogenic *E. coli.* With the use of PCR, pathogen genes were detected in six of the 23 colicinogenic *E. coli.* When the nonpathogenic strains were assessed for antibiotic resistance, four strains showed resistance to at least one antibiotic. The remaining set of 14 strains were evaluated for the presence of previously identified colicins. Seven colicins (B, E1, E2/E7, E7, Ia/Ib, K, and M) were detected. One half of the strains possessed multiple types of colicins. The most commonly detected colicins were B, E2/E7, and M, which were found in six strains each. DNA sequencing was also performed in order to classify the E2/E7 colicins separately from E7 colicins. The 14 colicinogenic *E. coli* also were evaluated for their ability to inhibit 10 different non-O157 pathogenic *E. coli.* Six of the colicinogenic *E. coli* were capable of inhibiting all 10 pathogens, and the remaining eight strains could each inhibit between six to eight of the pathogenic *E. coli.* This strain collection has great potential for inhibiting *E. coli* O157:H7 in cattle.

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Escherichia coli O157:H7 is a highly virulent foodborne pathogen that can cause severe gastrointestinal infections in humans. A large number of O157:H7 outbreaks are due to the consumption of meats, produce, water, and other foods that have been contaminated with cattle manure. Many studies have shown that a large number of cattle asymptomatically carry and shed this pathogen in their feces, and it is now well defined that these animals are the source of contamination *(1, 11).* Because of the widespread distribution of serotype O157:H7 in cattle populations, its control will require interventions at the farm level *(5).* As a result, various strategies are currently being investigated to reduce the prevalence of *E. coli* O157 in cattle.

A promising method for the control of foodborne pathogens in livestock is the feeding of beneficial bacteria, often referred to as probiotics, competitive exclusion, or direct-fed microbial products *(7).* Competitive exclusion cultures are typically mixtures of undefined microorganisms, whereas probiotics and direct-fed microbial products are individual or a combination of specific microbial strains. The development of probiotic cultures is an active research area, and a number of cultures have been tested with relative success. In poultry and swine, competitive exclusion and probiotic preparations have been used to eliminate *Salmonella* and enterotoxigenic *E. coli (14, 24),* but in cattle, only defined cultures have been investigated against *E. coli* O157:H7.

Several authors have identified bacteria with the potential ability to inhibit or exclude *E. coli* O157:H7 in the gastrointestinal tract of cattle. The first account of the use of probiotics to inhibit O157:H7 in cattle was by Zhao et al. *(33),* who identified several *E. coli* strains with the ability to inhibit *E. coli* O157:H7. The same probiotic *E. coli* were recently used to reduce the fecal shedding of enterohemorrhagic *E. coli* in neonatal calves *(34).* Ohya et al. *(25)* evaluated *Streptococcus bovis* and *Lactobacillus gallinarum* for the reduction of *E. coli* O157:H7 in calves. Brashears et al. *(3)* reported the use of lactobacilli in a cattle feeding study to reduce the fecal prevalence of serotype O157:H7. In our previous work, we reported the selection of 24 *E. coli* strains with the ability to inhibit O157:H7 *(29).* These strains were found to produce colicins, which are antimicrobial proteins active against *E. coli* or other enterobacteria.

Despite initial research efforts that indicated that probiotic bacteria could be used to reduce O157:H7 in cattle, little is known about the characteristics of these strains. Some important factors to consider when developing an anti-O157 mix for livestock include the presence of virulence factors, antibiotic resistance, type of antimicrobial proteins produced, and its specificity. Bacterial strains that have virulence genes or antibiotic resistance factors should not be fed to livestock for obvious reasons. These undesired traits would further flourish in the environment and potentially endanger the safety of livestock or even humans.

Another important consideration for a probiotic mix is the potential for the development of resistance to the product. For this reason, it is necessary to know the mechanism of action that the beneficial bacteria use against the targeted pathogen. The ideal probiotic mix would have several mechanisms of action to inhibit the pathogen; this would

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TABLE 1. *Primers used for the PCR detection of virulence factors and colicin genes and the designation of* E. coli *strains used as positive controls*

Gene	Primer sequence (5′–3′)	Product size (bp)	*E. coli* control strains (col plasmid)	Reference
stx_1	AGTTAATGTGGTGGCGAAGG GCCGGACACATAGAAGGAAA	804	ATCC 43895	This work
stx_2	ATCCTATTCCCGGGAGTTTACG GCGTCATCGTATACACAGGAGC	584	ATCC 43895	*6*
eae	CAGGTCGTCGTGTCTGCTAAA TCAGCGTGGTTGGATCAACCT	1,087	ATCC 43895	*13*
hly	AAGCCGGAACAGTTCTCTCA CACTTGCAGCTGTTGTCGAT	498	ATCC 43895	This work
LT	GGCGACAGATTATACCGTGC CCGAATTCTGTTATATATGTC	696	263 1362	*30*
STa	TCCGTGAAACAACATGACGG ATAACATCCAGCACAGGCAG	244	987 1362	*26*
STb	GCCTATGCATCTACACAATC TGAGAAATGGACAATGTCCG	278	987 1362	*26*
K88	ATCGGTGGTAGTATCACTGC AACCTGCGACGTCAACAAGA	601	1362 K88	*26*
K99	TGGGACTACCAATGCTTCTG TATCCACCATTAGACGGAGC	450	I92 1594	*26*
Colicin V	CACACACAAACGGGAGCTGTT CTTCCCGCAGCATAGTTCCAT	680	PAP222 (pColV-K270)	*17*
Colicin Ia/Ib	ACGTATTACAAATCCCGGTGC CTTTTTCCTTCAACAGGGCA	1,250	BZB2115 (pColIb-P90)	This work
Colicin K	GGTGATGTGAATAAACAGAAGG CAGCGTGACAAACAAAGG	792	BZB2116 (pColK-K49)	This work
Colicin B	AAGAAAATGACGAGAAGACG GAAAGACCAAAGGCTATAAGG	493	F16 U24	This work
Colicin M	CATCACCATCAACTAACTTACC CTCTTTACCAGAAAACATCG	737	PAP1 (pCHAP1)	This work
Colicin E1	TTTGAATGGTACTCCTGACGG GTTCCAGCAAGCAAGCTAAA	1,398	U24	This work
Colicin E7	ATGAGCGGTGGCGATGGACGC ACCTCGGTGAATATCAATATGTCG	1,705	BZB2110 (pColE7-K317)	*22*
E2/E3/E6/E7	ATGAGCGGTGGCGATGGACGC GCCCGGCCATTTGCCACATTCT	1,160	BZB2125 (pColE2-P9) BZB2106 (pColE3-CA38) BZB2109 (pC01E6-CT14) BZB2110	This work

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enhance the elimination of the pathogen and decrease the possibility for the development of resistance. It also would be potentially useful to determine whether probiotic *E. coli* could be used to control other pathogenic *E. coli* in livestock. The goal of this project was to further characterize the previously identified set of anti-O157 *E. coli* strains for their use as a direct-fed microbial mixture. This research was conducted to identify important characteristics of these strains, as well as their suitability for use in cattle.

MATERIALS AND METHODS

Strains and media. Strains of *E. coli* carrying previously characterized colicins (Table 1) were obtained from David Gordon (Australian National University, Canberra). Twenty-three strains of colicinogenic *E. coli* inhibitory to *E. coli* O157:H7 were identified from our previous work *(29)* are listed in Table 2. Ten strains of pathogenic *E. coli* were used in inhibition assays and as PCR-positive controls: O8:K87, O9:K103, O26, O101, O101:K30, O111, O113, O147:K87, O149, and O153. Six of those strains were obtained from Dr. Richard Isaacson (University of Minnesota, St. Paul). Serotype strains O113 and O153 were isolated in our laboratory from cattle feces. Strains O26 MC1156 and O111 MC1157 were obtained from Dr. John Besser at the Minnesota Department of Health. *E. coli* ATCC 25922 was used as a control strain for antibiotic testing.

Stock cultures of all strains were stored in vials containing 50% glycerol (Fisher Scientific, Pittsburgh, Pa.) at −50°C. Luria-Bertani (LB) broth (Difco, Sparks, Md.) and LA agar (LB broth with 1.5% agar) were used to cultivate strains at 37°C. Overlay assays were performed with LA soft agar (0.75% agar) along with LA agar plates containing mitomycin C (LAm) that was prepared with the addition of 0.25 mg mitomycin C (Sigma, Inc., St. Louis, Mo.) per liter.

Virulence factor gene detection. The colicinogenic *E. coli* were evaluated for the presence of nine different virulence factors:

TABLE 2. *Colicinogenic* E. coli *and their host source that had a detected virulence factor or antibiotic resistance; each colicinogenic* E. coli *strain was analyzed at least twice for the presence of virulence genes and antibiotic resistance, and those reported below were confirmed by independent experiments*[a]

Strain	Host source	Pathogen gene	Antibiotic resistance
G2	Deer	Hemolysin	NT
G15	Deer	STa	NT
H99	Human	ND	Amp
P37	Pig	Shiga toxin 1	NT
P39	Pig	Shiga toxin 1	NT
S5	Sheep	Hemolysin	NT
S7	Sheep	ND	Amp, Strep, Tet
S12	Sheep	ND	Tet
S24	Sheep	Hemolysin	NT
S33	Sheep	ND	Strep, Sul, Tet

[a] Amp, ampicillin; strep, streptomycin; Sul, sulfisoxazole; Tet, tetracycline; ND, not detected; NT, not tested.

shiga toxins (*stx*$_1$ and *stx*$_2$), intimin (*eae*), enterohemolysin (*hly*), heat-stable enterotoxins (STa and STb), heat-labile enterotoxin (LT), and adhesin fimbriae (K88 and K99). The detection of virulence properties was achieved with the use of PCR and specific primers (Table 1) for each gene. Bacterial cells used to derive the template were grown in LB overnight at 37°C. One milliliter of the broth was placed into a microcentrifuge tube and placed into a boiling water bath for 20 min. The microcentrifuge tubes were then placed on ice for 5 min and finally centrifuged at 14,000 × *g* for 2 min. Each 25-μl PCR reaction mixture contained 1 μl of cell template, 1 μM of each primer, 0.2 mM of each deoxynucleoside triphosphate (Fisher Scientific), 0.63 U Taq DNA polymerase (Promega, Inc., Madison, Wis.), 1× Taq buffer A, 1 mM $MgSO_4$, and 25 μg of bovine serum albumin (Promega). PCR reactions were performed with a Robocycler model Gradient 96 (Stratagene, Inc., La Jolla, Calif.) at 94°C for 2 min, followed by 35 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 1 min, followed by a final extension at 72°C for 5 min. Samples were then electrophoresed on a 0.8% agarose (Fisher Scientific) gel. The gels were stained with a 0.5 μg/ml ethidium bromide (ICN Biomedicals, Inc., Irvine, Calif.) solution, and destained with distilled water. Finally, a picture was taken with a Gel-Doc 8000 gel documentation system (UVP, Inc., Upland, Calif.).

Antibiotic susceptibility tests. Antibiotic susceptibility testing was performed with the disk diffusion assay specified by the NCCLS *(23)*. The susceptibility of the colicinogenic *E. coli* that had no virulence gene detected was evaluated against eight antibiotics: ampicillin 10 μg, cefazolin 30 μg, chloroamphenicol 30 μg, ciprofloxacin 5 μg, streptomycin 10 μg, sulfamethoxazole-trimethprim 25 μg, sulfisoxazole 0.25 mg, and tetracycline 30 μg (Becton Dickinson, Sparks, Md.). The colicinogenic *E. coli* and the control organism (*E. coli* ATCC 25922) were grown overnight on tryptic soy agar (Accumedia, Inc., Baltimore, Md.) plates at 37°C. Isolated colonies from these plates were used to make cell suspensions in tryptic soy broth (Accumedia) equivalent to a 0.5 McFarland turbidity standard. Next, a sterile cotton swab was dipped into a cell suspension and was used to streak a Mueller-Hinton agar (Difco) plate three times, with a 60° plate rotation between each streaking. The plates were allowed to dry for 3 to 5 min. Antibiotic disks were then placed on the plates, with no more than three discs placed onto a single plate. The plates were then incubated at 35°C for 16 to 18 h. Inhibition zones were measured and compared to the values set for sensitivity and resistance.

Siderophore assay. The colicinogenic *E. coli* were subjected to a siderophore assay to determine their ability to bind iron. A detection method developed by Schwyn and Neilands *(31)* was used, involving chrome azurol S (Sigma) agar plates. Overnight cultures of colicinogenic *E. coli* grown in LB were pelleted and resuspended in sterile water. Ten microliters of the resuspended cells were spot inoculated onto the chrome azurol S agar plates. The plates were incubated overnight at 37°C. A positive result for siderophore production was indicated by an orange halo surrounding the colony.

Colicin identification methods. The detection of colicin genes in the anti-O157 inhibitory *E. coli* was achieved with the use of the primers listed in Table 2. Primers against colicins Ia/Ib, B, K, M, and E2/E3/E6/E7 were designed on the basis of previously published sequences available in GenBank with Omiga 1.1.3 software (Oxford Molecular, Ltd., San Diego, Calif.). Colicins B, K, M, and V were detected with the same PCR mixture and reaction conditions as used for the detection of pathogen genes, described earlier. Colicins E1, E2, E3, E6, E7, and Ia/Ib were detected in 25-μl PCR reaction mixtures containing 1 μl of cell template, 1 μM of each primer, 0.4 mM of each deoxynucleoside triphosphate, 0.5 U Vent DNA polymerase (New England Biolabs, Beverly, Mass.), 1× Vent buffer, and 1 mM $MgSO_4$. This second set of colicins underwent PCR consisting of 94°C for 3 min, followed by 35 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 1 min and 42 s, followed by a final extension at 72°C for 5 min. Gel electrophoresis, staining, and documentation was performed as previously described.

Confirmation of colicin gene identification was performed by DNA sequencing. Amplified PCR products were extracted from agarose gels with the use of a commercial kit (Qiagen, Chartsworth, Calif.). Two-hundred fifty nanograms of PCR product and 3.2 pmol of primer were sent to the Advanced Genetic Analysis Center (University of Minnesota). The degree of nucleotide homology and the e-value of the amplified PCR products compared to previously characterized colicins was determined using GenBank BLAST.

Colicin sensitivity detection methods. Twenty-five microliters of overnight LB cultures of colicinogenic *E. coli* were spot inoculated onto LAm plates. After overnight incubation at 37°C, the agar media was flipped over. As a result, the *E. coli* colony was then on the bottom of the petri plate. Five milliliters of LA soft agar containing approximately 10^6 cells of the indicator strain was then used to overlay the plate. After overnight incubation at 37°C, the plates were examined for clear inhibition zones. Observed inhibition zones were measured and recorded.

RESULTS

When the 23 O157:H7-inhibiting colicinogenic *E. coli* were assessed for the presence of virulence-associated factors, six strains were detected as having a virulence gene (Table 2). One deer and two sheep isolates (G4, S5, and S24) were found to possess enterohemolysin. Another deer isolate (G15) was found to have STa. Shiga toxin 1 was detected in both swine strains (P37 and P39). None of the strains were detected as carrying genes for shiga toxin 2, intimin, LT, STb, K88, or K99. The six virulence factor–positive colicinogenic *E. coli* were eliminated from further testing.

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TABLE 3. *Strain identification and origin source of colicinogenic* E. coli *and their colicin genes that were detected with PCR; each colicinogenic* E. coli *strain was analyzed at least twice for the presence of all seven colicins, and those reported below were confirmed by two or more independent experiments*

Strain	Host source	No. of colicins	Colicins detected
F4	Cat	2	B, M
F16	Cat	4	B, E1, Ia/Ib, M
U24	Duck	4	B, E2/E7, Ia/Ib, M
H30	Human	1	E7
H31	Human	1	E7
H43	Human	1	Ia/Ib
H70	Human	2	B, M
B15	Cattle	1	E2/E7
B18	Cattle	1	E2/E7
B23	Cattle	3	B, Ia/Ib, M
B34	Cattle	1	E2/E7
S12	Sheep	1	E2/E7
S34	Sheep	2	E2/E7, K
S35	Sheep	3	B, K, M

The remaining 17 colicinogenic *E. coli* were evaluated for their sensitivity to eight different antibiotics. Four strains were found to be resistant to at least one antibiotic (Table 2). The human strain H99 was resistant to ampicillin and the sheep isolate S12 was tetracycline resistant. Strain S7 was resistant to ampicillin, streptomycin, and tetracycline, whereas S33 was resistant to streptomycin, sulfisoxazole, and tetracycline. Strains S7 and S33 were eliminated from further characterization because of their multiple antibiotic resistance, and H99 was discarded because of its ampicillin resistance. When the colicinogenic *E. coli* were grown on chrome azurol S agar plates, each produced a characteristic orange halo surrounding the bacterial growth. This indicated that all of the strains produced siderophores for iron binding capability.

The selected 14 colicinogenic *E. coli* were then tested by PCR for the presence of previously characterized colicins. The most frequently detected colicins included B, E2/E7, and M, which were found in six strains each (Table 3). Colicin Ia/Ib was found in four strains, E7 in two strains, K in two strains, and E1 in one strain. One half of the strains were determined to possess multiple colicins. Strains F16 and U24 carried as many as four distinct colicins. Six of the seven strains that contain only one detected colicin were found to possess either E2/E7 or E7. Colicins B and M were always detected together in the colicinogenic strains.

Because of the lack of colicinogenic *E. coli* strains as positive controls for colicins B and E1, DNA sequencing was used to confirm identity. Amplified colicin gene products for colicin B from strains F16 and U24, as well as colicin E1 from strain F16, were sequenced. In all cases, the homology of the amplified B and E1 colicins was at least 99% when compared with previously characterized B and E1 colicins. These strains were then used as positive control organisms for the PCR detection of colicins B and E1.

PCR detection could not distinguish between colicins E2/E7 and E7 because of their similar homology. Therefore, DNA sequencing was performed on the 5′ and 3′ end of the eight strains that had this PCR product. Sequence data was compared to three previously characterized colicins by GenBank: E2, Hu194, and E7. The resulting homology and e-values can be found in Table 4. Six strains were found to be identical or nearly identical to Hu194, indicating an E2/E7-type colicin, whereas the two other strains, H30 and H31, were found to be most closely related to colicin E7.

The 14 colicinogenic *E. coli* were evaluated for their

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TABLE 4. *Colicinogenic* E. coli *possessing an E2/E7 or E7 colicin and how those colicins compared to previously sequences E2, Hu194, and E7 colicins from GenBank; each sequence was determined in duplicate from two separate PCR reactions*

Strain	Gene region	Nucleotide homology [no. of identical/total no. of nucleotides (% homology), e-value]		
		E2	Hu194	E7
U24	5′ end	162/182 (87), 5e-49	612/612 (100), 0.0	593/612 (96), 0.0
	3′ end	656/696 (94), 0.0	696/697 (99), 0.0	482/536 (89), e-179
H30	5′ end	167/188 (88), 3e-50	707/707 (100), 0.0	684/710 (95), 0.0
	3′ end	452/515 (87), 3-145	483/536 (90), 0.0	660/660 (100), 0.0
H31	5′ end	158/177 (89), 2e-48	685/685 (100), 0.0	662/688 (96), 0.0
	3′ end	452/515 (87), e-145	483/536 (90), 0.0	680/680 (100), 0.0
B15	5′ end	167/188 (88), 3e-50	618/618 (100), 0.0	599/621 (96), 0.0
	3′ end	699/749 (93), 0.0	756/756 (100), 0.0	545/599 (90), 0.0
B18	5′ end	167/188 (88), 3e-50	711/711 (100), 0.0	688/714 (96), 0.0
	3′ end	710/763 (93), 0.0	770/770 (100), 0.0	553/607 (91), 0.0
B34	5′ end	167/188 (88), 4e-50	754/754 (100), 0.0	729/757 (96), 0.0
	3′ end	696/748 (93), 0.0	748/748 (100), 0.0	547/601 (91), 0.0
S12	5′ end	167/188 (88), 3e-50	731/732 (99), 0.0	705/733 (96), 0.0
	3′ end	655/696 (94), 0.0	697/697 (100), 0.0	483/536 (90), 0.0
S34	5′ end	165/188 (87), 2e-45	676/699 (96), 0.0	684/702 (96), 0.0
	3′ end	666/708 (94), 0.0	713/715 (99), 0.0	504/559 (90), 0.0

TABLE 5. *Sensitivity of pathogenic* E. coli *serotypes to O157:H7-inhibiting colicinogenic* E. coli

Strain	Inhibition of pathogenic *E. coli* serotypes[a]									
	O8:K87	O9:K103	O26	O101	O101:K30	O111	O113	O147	O149	O153
F4	++	++	++	++	++	++	++	++	++	++
F16	++	++	++	++	++	++	++	++	++	++
U24	++	++	++	++	++	++	++	++	+/++	++
H30	++	++	++	++	++	++	−	++	−	−
H31	++	++	++	++	++	++	−	++	−	−
H43	++	++	++	++	++	+/++	++	++	++	++
H70	++	++	++	++	++	++	++	++	+/++	++
B15	++	++	++	++	+	−	−	++	−	−
B18	++	++	++	++	+	−	−	++	−	−
B23	−/+	++	++	++	+	++	++	−	−	++
B34	++	++	++	++	+	−	−	++	−	−
S12	++	++	++	++	−	−	−	++	++	−
S34	++	++	++	++	+	+/++	−	++	++	−
S35	++	++	++	++	+	++	++	++	++	++

[a] Observations were collected from two independent experiments. ++, clear inhibition zones 15 mm or more in diameter; +, clear inhibition zones less than 15 mm in diameter; −, no inhibition zones; −/+ or +/++, replicates showed different zones sizes between two independent trials.

ability to inhibit 10 different non-O157 pathogenic *E. coli* (Table 5). Six of the colicinogenic *E. coli* were capable of inhibiting all 10 pathogens. The remaining eight strains could each inhibit between six to eight of the pathogenic *E. coli.* The six strains that could only inhibit six or seven of the pathogenic *E. coli* were found to possess only one detected colicin, either E2/E7 or E7. The two human isolates possessing only colicin E7 (H30 and H31) had identical inhibition patterns. Similarly, three bovine isolates (B15, B18, and B34) that were detected as having only colicin E2/E7 had matching inhibition results.

DISCUSSION

The use of beneficial bacteria to inhibit or exclude *E. coli* O157:H7 in cattle is a promising method to control this foodborne pathogen. However, little is known regarding the characteristics of the strains that compose anti-O157 bacterial mixes. One important set of characteristics that probiotic bacteria should be screened for is the presence of virulence factors. The feeding of bacteria to livestock for beneficial reasons could be detrimental, if the fed bacteria themselves possess pathogenic features. DebRoy and Maddox *(9)* have suggested a number of virulence factors that *E. coli* strains should be screened for. In the present study, the nine virulence factors were selected on the basis of those typically present in enterohemorrhagic and enterotoxigenic *E. coli* serotypes. When the initial set of *E. coli* was screened for pathogenic factors, 26% of the strains had at least a virulence gene. These strains should not be used in livestock to avoid the spread of virulence factors in the environment.

The emergence of antibiotic-resistant bacteria has become a major public health concern *(15).* The extensive use of antibiotics in agriculture applications might lead to the selection of resistant strains that could proliferate and pass their resistance factors to human pathogens. Subsequently, the U.S. Food and Drug Administration (FDA) has proposed an outline of criteria for evaluating products intended for use in food-producing animals *(8).* Antimicrobial products are being classified into one of three categories on the basis of the drug's relevance to human health care.

The use of probiotic bacteria with antibiotic-resistant factors in livestock would be undesirable because the resistance genes could be transferred to other microorganisms in the gastrointestinal tract and the environment *(15).* Brashears et al. *(4)* found that 68% of their anti-O157 lactic acid bacteria were multiple antibiotic resistant. In this study, 2 of 17 strains (12%) were multiple antibiotic resistant, and 2 other strains were resistant to a single antibiotic. Therefore, the multiple antibiotic–resistant bacteria were removed from further characterization. Although strain H99 was only resistant to ampicillin, this isolate was eliminated from further characterization because the FDA classifies this antibiotic as a Category II antimicrobial. This designation indicates that the use of this strain would require FDA approval on the basis of antibiotic resistance data. In contrast, strain S12 was not eliminated even though it was tetracycline-resistant because it is not listed among FDA's Category I and II antimicrobials *(8).*

The gastrointestinal tract is an environment in which microorganisms compete for limited resources. One such resource that bacteria require for growth is iron *(16).* In order to acquire it, bacteria produce siderophores that sequester iron. Studies have shown that *E. coli* possessing siderophores have an increased advantage for the colonization of the gastrointestinal tract *(10).* The remaining 14 colicinogenic strains of this study were able to produce siderophores. This result was not unexpected because Maurer et al. *(20)* found that 96% of *E. coli* strains for a chicken competitive exclusion product had the ability to produce siderophores.

The ability to successfully use probiotics against a targeted organism can be mediated on one of three modes of action: site attachment, nutrient competition, or the produc-

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tion of antimicrobial substances *(12).* The ability of some strains of *E. coli* to inhibit other *E. coli* is accomplished by the production of antimicrobial proteins called colicins. Surveys have shown that approximately 30% of all *E. coli* can produce a colicin *(28).* Colicins can be classified on the basis of their mode of action and internalization pathways *(18).* Modes of action include formation of membrane pores, DNA cleavage, RNA degradation, and inhibition of peptidoglycan synthesis.

Several studies have been performed to assess the ability of colicins to inhibit O157:H7 *(2, 21, 29).* In the first reported paper of using anti-O157 *E. coli* in cattle, there was no attempt to identify the anti-O157 compounds produced *(33).* Lyon and Olson *(19)* reported the isolation of colicin ECL 12 that was able to inhibit serotype O157. The authors partially identified this colicin, finding that it had DNase activity and a molecular mass of 65,000. The set of strains used in the present report were previously selected on the basis of their ability to inhibit O157:H7 strains mediated by the production of colicin proteins *(29).* However, the type of colicins and the presence of undesirable characteristics of each of those strains were not known.

This strain collection was found to produce seven different types of colicins representing three different modes of action. Colicins B, E1, Ia/Ib, and K are pore formers, colicins E2 and E7 are DNases, and colicin M inhibits peptidoglycan synthesis. A successful strategy of *E. coli* O157:H7 inhibition based on colicinogenic *E. coli* should involve more than one type of colicin. The dependence of one colicin type would increase the chances for the selection of colicin-resistant O157:H7 serotype mutants. A successful long-term strategy for *E. coli* O157:H7 reduction would entail the use of multiple colicins with different mechanisms of action while reducing the possible selection of resistant mutants. This is the first report that addresses the potential for resistance in the development of a probiotic bacterial mixture.

According to Riley and Gordon *(28),* the frequency of colicin type varies by the animal species. Colicins M and V appeared to be more frequently found in chickens than in humans. As a result, *E. coli* naturally present in poultry would likely be more resistant to these colicins because they tend to occupy the same niche. The probiotic *E. coli* strains reported by Zhao et al. *(33)* were isolated from cattle, and they were selected on the basis of their inhibitory activity against *E. coli* O157:H7. Interestingly, the authors reported that from 1,200 bacterial strains tested, only 18 were inhibitory against a mixture of five O157:H7 strains. This low level of colicinogeny could suggest that *E. coli* O157:H7 might be more resistant to specific types of colicinogenic *E. coli* normally present in cattle. In the present study, three of the four bovine isolates only produced E2/E7-type colicins. The selection of anti-O157 *E. coli* from different animals increased the number of *E. coli* that produced a variety of colicins.

At least three separate studies have reported that colicins B, E1, E2, E7, K, and Ia/Ib can inhibit *E. coli* O157:H7, but the degree and extent of inhibition appears to be variable *(2, 21, 29).* Murinda et al. *(21)* found that colicin K could inhibit 11 of 11 O157:H7 strains, but Schamberger and Diez-Gonzalez *(29)* observed that colicin K was able to inhibit four of seven O157:H7 strains, whereas Bradley et al. *(2)* reported that colicin K could not inhibit any of the 20 *E. coli* O157:H7 strains tested. These differences could be due to varying colicin sensitivities of O157:H7 strains used or different lab techniques, but this variability stresses the importance of a multi-colicinogenic probiotic culture.

An interesting colicin that has been detected in this collection is the E2/E7 type, which was found in six strains. This colicin appears to be a hybrid between colicins E2 and E7 that might have been the result of homologous recombination. The 5′ end of the colicin gene is very homologous to colicin E7. But at the 3′ end, the colicin gene was very similar to colicin E2. Tan and Riley *(32)* reported recombinant E2/E7 colicins from *E. coli* recovered from Australian feral mice and an *E. coli* strain isolated in France over 50 years ago, referring to them as E2 type 2 colicins. Recently Nandiwada et al. *(22)* characterized the anti-O157 colicin Hu194, which was an E2/E7 hybrid. This colicin type might be very effective against O157:H7, considering that in the present study, it was the only detected colicin in four of the six strains that possessed it.

Colicins are single-subunit proteins of 30 to 70 kDa that have four distinct domains *(27).* The N-terminal region is involved in translocation, and the central domain functions in receptor recognition and binding. The C-terminal region contains the catalytic/killing activity as well as the binding portion to the immunity protein. From the sequence data, the E2-similar 3′ end would constitute the colicin's killing activity, whereas the E7-similar 5′ end would be involved with translocation across the cell membrane. On the basis of our previous report that E7 was more effective against O157:H7 strains than E2 and the observation that E7-like colicins were the most common colicin detected in this collection, it appeared that the E7-type receptor region might be uniquely designed against this pathogen.

E. coli serotype O157:H7 attracts significant attention as a virulent foodborne pathogen; however, other non-O157 pathogenic *E. coli* are also recognized as major public health hazards. The ability of anti-O157 *E. coli* to inhibit other non-O157 pathogenic *E. coli* could have potential applications to control other shiga toxin-producing and enterotoxigenic *E. coli* serotypes, such as O26, O111, K88, K99, and F18. This present set of diverse colicinogenic *E. coli* were assessed for inhibitory activity against 10 non-O157 pathogenic *E. coli,* and 6 of the 14 strains were able to inhibit all 10 strains. The ability of some of these colicinogenic *E. coli* to inhibit a range of *E. coli* might be in part due to their ability to produce multiple colicins.

The use of colicinogenic *E. coli* to reduce *E. coli* O157:H7 in cattle is a very promising intervention. Besides this strain set's strong inhibition of O157:H7, the lack of virulence genes and antibiotic resistance would permit the use of these strains in livestock. The presence of various colicin types would enhance the ability of this strain set to inhibit serotype O157:H7 and decrease the possibility of resis-

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tance. The probiotic potential of this collection of strains will be further evaluated by in vivo experimental trials.

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