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Research Note

Acquisition of Antibiotic Resistance Plasmids by Enterohemorrhagic *Escherichia coli* O157:H7 within Rumen Fluid

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ABSTRACT

The emergence of antibiotic resistance among important foodborne pathogens like *Escherichia coli* O157:H7 has become an important issue with regard to food safety. In contrast to the case for *Salmonella,* antibiotic resistance has been slow in its development in *E. coli* O157:H7 despite the presence of mobile antibiotic resistance genes in other *E. coli* organisms that inhabit the same animal host. We set out to determine if rumen fluid influences the transfer of plasmid-mediated, antibiotic resistance to *E. coli* O157:H7. A commensal *E. coli* strain from a dairy cow was transformed with conjugative R plasmids and served as the donor in matings with naladixic acid–resistant *E. coli* O157:H7. R plasmids were transferred from the donor *E. coli* strain to *E. coli* O157:H7 in both Luria-Bertani (LB) broth and rumen fluid. R plasmids were transferred at a higher frequency to *E. coli* O157:H7 during 6 h of incubation in rumen fluid at rates comparable to those in LB broth, indicating that conditions in rumen fluid favor the transfer of the plasmids to *E. coli* O157. This finding suggests that the cow's rumen is a favorable environment for the genetic exchange of plasmids between microflora and resident *E. coli* O157:H7 in the bovine host.

Enterohemorrhagic *E. coli* is the leading cause of hemorrhagic colitis and hemolytic uremic syndrome in the United States and Canada. The transmission of enterohemorrhagic *E. coli* occurs through the consumption of improperly cooked beef products or unpasteurized milk. Ruminants are important reservoirs for *E. coli* O157:H7 *(21).*

The emergence of drug resistance in foodborne pathogens has made antibiotic resistance an important food safety issue *(7, 15).* Epidemiological studies suggest an association between the use of antibiotics in animals and the isolation of resistant bacteria from the same animals *(6, 7).* Once antibiotic pressure has been introduced into an environment, antimicrobial resistance quickly develops and spreads *(20),* with antibiotic-resistant bacteria becoming the dominant component of the gut flora *(13, 17).*

Antibiotic resistance due to the acquisition of conjugative R plasmids *(27)* has been detected in *E. coli* O157:H7 *(15).* Although epidemiological studies suggest that the administration of antibiotics to animals is partially responsible for the emergence of resistance, there is little direct experimental information to indicate the frequency of these events. Also, the extent to which commensal bacteria serve as reservoirs for drug resistance acquired by these human pathogens in their ruminant host is unknown. In this study, we examine the feasibility of plasmid transfer between commensal *E. coli* and antibiotic-susceptible *E. coli* O157:H7 within rumen fluid.

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MATERIALS AND METHODS

Conjugal transfer of antibiotic resistance plasmids within rumen fluid. A commensal *E. coli* strain (MZ10) was isolated from the rumen of a dairy cow by a standard isolation and bacterial identification scheme. This *E. coli* isolate was sensitive to several antibiotics, including naladixic acid. This bovine *E. coli* isolate was transformed by electroporation *(9)* with the mobilizable, broad-host-range plasmid pMMB67 (encoding ampicillin resistance *(12)*) and the conjugative plasmid pRK2013 (encoding kanamycin resistance *(10)*). The *E. coli* donor is ampicillin- and kanamycin-resistant but is susceptible to naladixic acid. This transformant served as the donor in matings. A naladixic acid–resistant *E. coli* O157:H7 strain (NA932) served as the recipient in this study *(3).* Standing overnight cultures of *E. coli* O157:H7-NA932 (6×10^8 CFU/ml) and *E. coli* MZ10 (9×10^8 CFU/ml) (containing plasmids pMMB67 and pRK2013) grown in Luria-Bertani (LB) broth were added to LB broth (0.5 ml) or rumen fluid (0.5 ml) at a 1:1 ratio and incubated at 37°C for 6 or 24 h. Broth mating was also carried out with the recipient and the donor alone. Ruminal contents (50 ml) were collected via a rumen cannula from a calf *(25).* Mixed suspension (100 μl) was plated on sorbitol-MacConkey agar (SMAC) with naladixic acid (N) alone, ampicillin (A) and kanamycin (K), N with A, and N with A and K. Each antibiotic was included in SMAC at a final concentration 50 μg/ml. Transconjugants, the recipient *E. coli* O157:H7 organisms that had obtained either plasmid, were obtained on SMAC

TABLE 1. *Transfer of antibiotic resistance to* E. coli *O157:H7 from a bovine rumen* E. coli

	LB broth[a]				Rumen fluid[a]			
Trial	Recipient *E. coli* O157[b]	*E. coli* O157 + both plasmids (frequency)	*E. coli* O157 + pRK2013 (frequency)	Donor *E. coli* MZ10[c]	Recipient *E. coli* O157[b]	*E. coli* O157 + both plasmids (frequency)	*E. coli* O157+ pRK2013 (frequency)	Donor *E. coli* MZ10[c]
Trial 1								
6 h	0	7 (1×10^{-8})	3 (5×10^{-9})	0	0	66 (1×10^{-7})	29 (6×10^{-8})	0
24 h	0	2,000 (4×10^{-6})	1,200 (2.4×10^{-6})	0	0	184 (4×10^{-7})	16 (3×10^{-8})	0
Trial 2								
6 h	0	6 (1×10^{-8})	12 (2×10^{-8})	0	0	751 (2×10^{-6})	532 (1×10^{-6})	0

[a] Plate counts (CFU/100 μl of mixed suspension) for broth mating on sorbitol-MacConkey agar with N + A ± K.
[b] Recipient *E. coli* O157 strain is resistant to N (>50 μg/ml) but susceptible to A and K (50 μg/ml).
[c] Donor *E. coli* MZ10, with plasmids pRK2013 and pMMB67, is resistant to both A and K but susceptible to N.

with N + A ± K. Cells were plated on SMAC with N or with A + K to ascertain the viability of either the recipient or the donor following mating in rumen fluid. Plating of the recipient on SMAC with A + K and of the donor on SMAC with N alone was carried out to assess spontaneous mutation to resistance to either drug arrangement. The transfer rate was calculated as the number of transconjugants divided by the number of recipient cells present in broth mating.

Characterization of *E. coli* O157:H7 transconjugants. Probes for enterohemorrhagic *E. coli stx1* and *stx2* were created by polymerase chain reaction by using digoxigenin-labeled nucleotides according to the procedure of Maurer et al. *(18)*. The PCR reaction mix consisted of 0.2 mM dNTPs, 2.0 mM $MgCl_2$, 1× PCR buffer (50 mM Tris, pH 7.4), bovine serum albumin (0.25 mg/ml), 50 pmol of forward and reverse PCR primers, and 0.5 U of *Taq* DNA polymerase (Boehringer Mannheim, Indianapolis, Ind.). PCR primers for *stx1* (F: GACTTCTCGACTGCAAAGACGTATG; R: TTATCCCCTGAGCCACTATCAATC) and *stx2* (F: AATGGGTACTGTGCCTGTTAC TGG; R: TGCTGTCCGTTGTCATGGAAAC) amplified PCR products of 128 and 384 bp, respectively. The program parameters for the Idaho Technology Rapidcycler (Idaho Technology, Idaho Falls, Idaho) were 94°C for 0 s, 55°C for 0 s, and 72°C for 15 s for 30 cycles. DNA products from PCR were analyzed by gel electrophoresis. DNA-DNA hybridizations and washes were performed at 60°C as described by Sambrook et al. *(22)*. Hybridization was detected using the anti-digoxigenin antibody-alkaline phosphatase conjugate provided with the Genius System (Boehringer Mannheim).

RESULTS AND DISCUSSION

Drug resistance was detected in *E. coli* O157:H7 Na932 after incubation with the antibiotic-resistant bovine *E. coli* (MZ10) (Table 1). Reasonable numbers of transconjugants were obtained, and both the donor (MZ10) and the recipient (Na932) were recovered in large numbers 24 h after inoculation into the rumen fluid (Table 1). Colonies on plates with N + K ± A represent *E. coli* O157:H7 transconjugants. No colonies appeared on plates with N + A + K for either the donor only or the recipient only (controls). Colonies picked from N + K + A plates tested positive by PCR for *stx1* and *stx2* genetic markers for *E. coli* O157:H7 and contained the antibiotic resistance plasmids pMMB67 and pRK2013 (data not shown). The small numbers of transconjugants was not attributed to a decrease in the viability of either the donor or the recipient during 6 or 24 h of incubation, as evident from the high plate counts obtained from broth mating on SMAC with N or with A + K. On the basis of the number of recipient cells inoculated into the rumen fluid in trial 2, the transfer rate was 2×10^{-6} transconjugants per recipient cell. The transmission of resistance plasmids to *E. coli* O157:H7 appeared to occur more readily in rumen fluid than in LB broth during the short (6-h) mating period. Increasing the mating period from 6 to 24 h in the rumen fluid did not appear to result in an increase in the rate of transfer of plasmids to *E. coli* O157:H7. The transfer of genetic elements such as plasmids between microflora and enterohemorrhagic *E. coli* appears to occur quite readily in rumen fluid.

Genetic exchange of drug resistance has been documented through epidemiological observations *(2, 19, 20, 24)* and in experimental models *(5, 8, 14, 23)*. Only one in vivo study has directly addressed the potential for the transfer of drug resistance from commensals to pathogens (albeit for a veterinary pathogen) resident in the same animal host *(16)*. Here we have demonstrated the feasibility of the genetic transfer of antibiotic resistance to *E. coli* O157:H7 in a complex microbial consortium representative of the ecosystem of the rumen.

Despite the complex nature of the rumen fluid, the plasmid donor encountered *E. coli* O157:H7 and transferred its resident plasmids to its new recipient host at a frequency exceeding that observed for broth mating in LB broth. While infertility is due to less-than-optimum growth rates because of anaerobic conditions (bile salts and fatty acids that impede transfer *(11)*), we did not find similar physiological factors to be impediments to plasmid transfer.

The acquisition of antibiotic resistance plasmids can result in an ecological disadvantage for the transconjugant if it has to compete with the resident microflora *(1)*. We do not know if R plasmids might also present *E. coli* O157:H7 with a similar hardship in the bovine gastrointestinal tract. It is also possible that R plasmids are unstable in *E. coli* O157 because of plasmid incompatibility with the O157 virulence plasmid *(4)*. A limited repertoire of R plasmids belonging to incompatibility groups compatible with

the O157 virulence plasmid may explain the slow and limited emergence of resistance in *E. coli* O157:H7.

The slow development in *E. coli* O157:H7 of resistance to antimicrobial agents is perplexing, since other *E. coli* strains that inhabit the same animal host have acquired resistance to multiple antibiotics *(26).* We do know that coliforms, including *E. coli* O157:H7, are present in the rumen in small numbers *(3).* If the reservoir of antibiotic resistance genes and plasmids is narrow, the probability of the transfer of plasmids to *E. coli* O157:H7 is low, since transfer is contingent on physical cell-to-cell contact.

Our findings indicate that it is not the environment of the rumen that precludes the transfer of resistance to *E. coli* O157:H7. Why is there such slow and limited emergence of resistance in *E. coli* O157:H7 in nature? Long-term epidemiological studies on feedlot cattle, as well as other animal studies, may provide us with the clues necessary to solve this perplexing problem.

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