Evaluation of the control of pathogen load by an anti-Salmonella bacterium in a herd of cattle with persistent Salmonella infection

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Objective—To determine whether an anti-Salmonella bacterium is involved in control of pathogen load in persistently infected cattle herds.

Animals—24 Holstein calves experimentally infected and 39 Holstein cows naturally infected with Salmonella spp.

Procedures—An Escherichia coli (designated as P8E5) that possessed anti-Salmonella activity was isolated from Salmonella-negative bovine feces obtained from a herd with endemic Salmonella infection. In vitro analysis involved enumerating Salmonella enterica serovar Typhimurium coincubated with E coli P8E5. In vivo analysis involved coadministration of Salmonella spp and E coli P8E5 or an E coli control strain to neonatal Holstein calves. Fecal samples were collected on multiple days after inoculation, and quantitative PCR assay was performed by use of Salmonella-specific primers.

Results—E coli P8E5 reduced viability of Salmonella spp in vitro. Shedding of Salmonella organisms was diminished in calves administered F coli P8E5, whereas the control strain of E coli had no effect on shedding of Salmonella organisms.

Conclusions and Clinical Relevance—In this study, an E coli strain was identified that possessed bacteriocin-like activity and was able to decrease viability of Salmonella organisms in vitro and in vivo. Therefore, it is possible that this organism could be representative of native microbiota that dampen Salmonella spp in persistently infected cattle herds. (Am J Vet Res 2000;70:92-98)

Salmonella spp are common food-borne pathogens throughout the world. These organisms are ubiquitous among animals and are carried in the intestinal tract and associated organs. Certain Salmonella spp do not cause overt disease in their host, although fecal shedding perpetuates the fecal-oral transmission cycle. This problem is especially evident in some herds in which cattle are persistently infected with Salmonella spp. Within these herds, some cattle are carriers, whereas other cattle appear to be resistant to colonization despite constant exposure to the pathogen. One hypothesis for this phenomenon is that some animals harbor microflora that effectively excludes Salmonella organisms or other microorganisms from the intestinal tract; this concept is known as competitive exclusion. Competitive exclusion bacteria are believed to prevent pathogen colonization through antibacterial factors, immune exclusion, or competitive adhesion.

Intestinal bacteria produce a variety of compounds that have antibacterial properties. One group of these compounds is bacteriocins, which are antimicrobial peptides produced by bacteria for the purpose of inhibiting adjacent bacteria. When administered directly to cattle, bacteriocins or bacteriocin-producing bacteria reduce colonization or shedding (or both) of food-borne pathogens. The objective of the study reported here was to determine whether an anti-Salmonella bacterium could be cultured from fecal samples collected from a herd of cattle with a heterogeneous history of shedding of Salmonella organisms (ie, some cattle were persistent shedders, whereas others remained free of Salmonella organisms, possibly because of microfloral differences that mitigated the repeated exposure to the pathogen).

Materials and Methods

Sample population—Fecal samples were obtained from 39 Holstein cows from a herd with persistent Salmonella infection. In addition, 24 neonatal Holstein calves were experimentally infected with Salmonella enterica serovar Typhimurium.
bull calves were used as a method to evaluate colonization of adult cattle. Calves were 1 to 2 weeks old, weighed approximately 45 kg each, and were fed a diet of milk replacer. Animal experiments were approved by the Animal Care and Use Committee at the National Animal Disease Center (protocol No. 3462).

**Bacterial cultures, growth conditions, and plasmids**—Several bacterial strains and plasmids were used in the study.\(^\text{20-22a}\) (Appendix). All bacterial cultures were maintained on plates consisting of Lennox agar\(^a\) containing the appropriate antimicrobial, and cultures were grown overnight in Lennox broth\(^b\) containing antimicrobials. Unless otherwise stated, broth cultures were incubated at 37°C with shaking at 200 revolutions/min. Ampicillin\(^4\) and zeocin\(^4\) were used at 32 μg/mL and 25 μg/mL, respectively. Plasmid DNA was purified by use of a DNA purification kit in accordance with the manufacturer's instructions. For coincidence experiments, electrocompetent Escherichia coli P8E5 were electroporated\(^5\) (25 μF 2.5 kV 200 Ω) with pSM340 encoding GFP. Transformation of pSM340 conferred green fluorescence to bacterial colonies when exposed to UV light at 300 nm.

*Salmonella enterica* serovar Typhimurium strain 968G was chosen for in vitro studies because of its multiresistance pattern (ie, this strain can be distinguished from *E. coli* strains and any other microbes in the assay). *Salmonella enterica* serovar Typhimurium strain BJ68\(^2\) was chosen for in vivo studies because it colonizes calves but does not cause overt systemic disease (ie, extended in vivo studies can be performed because of a lack of clinical disease).

**Anti-Salmonella activity**—Fecal samples were initially collected from 19 Holstein cows from a farm in Minnesota. Of these 19 cows, 11 had multiple episodes of salmonellosis, and the other 8 had no history of salmonellosis or *Salmonella* shedding (2 successive annual culture-negative results). An aliquot (2 g) of each of the 19 fecal samples was inoculated into 50 mL of Lennox broth and incubated aerobically overnight at 37°C. Supernatants, which represented the collection of bacteria in a given fecal sample, were collected after centrifugation (1,500 X g for 15 minutes). Bacteria-free supernatants were pipetted into 96-well plates (100 μL/well) and coincubated with an equal volume of *Salmonella Typhimurium* strain 968G (approx 2.5 X 10^9 CFUs) or Lennox broth (negative control sample). The 96-well plates were incubated overnight at 37°C with shaking at 200 revolutions/min. The supernatant of 1 fecal sample after growth on plates containing *Salmonella* Typhimurium strain 968G (approx 2.5 X 10^9 CFUs) in fresh 96-well plates, which were incubated overnight at 37°C with shaking at 200 revolutions/min. Anti-Salmonella activity was visually determined by the lack of *Salmonella Typhimurium* 968G growth (apparent OD\(_{600}\) = 0) in the wells. A specific colony (designated as P8E5) was found to possess anti-Salmonella activity (no apparent growth of *Salmonella* spp in the well) and was identified by use of a commercial kit in accordance with the manufacturer's instructions.

**E coli P8E5 in vitro anti-Salmonella assays**—Escherichia coli P8E5 (approx 2.5 X 10^9 CFUs) and *Salmonella Typhimurium* 968G (approx 2.5 X 10^9 CFUs) were coincubated in Lennox broth overnight at 37°C. The inoculum was then transferred to agar plates and incubated overnight, and viability counts were then determined for both bacteria by use of Lennox broth agar (green colonies for *E. coli* P8E5) or Lennox broth–zeocin agar (for *Salmonella Typhimurium* 968G). This process was repeated every 24 hours for 8 days. Cultures of *E. coli* P8E5 and *E. coli* Top10 (negative control sample) were centrifuged at 15,000 x g for 10 minutes; supernatants were sterilized by use of a filter. Cultures of *Salmonella Typhimurium* (strains BJ68\(^1\) and 968G), *S. enterica* serovar Dublin, or *S. enterica* serovar Newport were placed into separate test tubes (2 mL/tube; approx 2 X 10^10 CFUs/tube), and 2 mL of *E. coli* P8E5 or *E. coli* Top10 supernatant (derived from approx 2 X 10^10 CFUs) was added to the tubes, which were then incubated overnight at 37°C with shaking at 200 revolutions/min. Inocula were then assayed for turbidity by use of spectrophotometric analysis at 600 nm.

**P8E5 in vivo anti-Salmonella assays**—Twelve calves (3 calves/pen) were orally administered 10^10 CFUs of *Salmonella Typhimurium* BJ68 in gelatin capsules on day 0. On day 3, 6 calves were orally administered 3 X 10^10 CFUs of *E. coli* P8E5, whereas the other 6 calves were orally administered 3 X 10^10 CFUs of *E. coli* Top10. Fecal samples were collected on days 3, 8, 11, 15, and 18 after *Salmonella* inoculation. On day 22, calves were euthanatized by administration of xylazine\(^6\) (0.45 mg/kg, IM) followed by pentobarbital\(^7\) (1.2 mg/kg, IV), and luminal contents of the intestines were collected (3 samples/calf).

In another experiment, *E. coli* were administered before oral challenge exposure with *Salmonella* organisms. Specifically, 6 calves were orally administered 3 X 10^10 CFUs of *E. coli* P8E5, and 6 other calves received 3 X 10^10 CFUs of *E. coli* Top10 (day –3). Three days later, all 12 calves were challenged exposed by oral administration of 10^9 CFUs of *Salmonella Typhimurium* BJ68 (day 0). Fecal samples were collected on days 3, 5, and 7 after inoculation with *Salmonella* organisms. On day 9, calves were euthanatized, and luminal contents of the intestines were collected at stated previously. Colonization of *E. coli* P8E5(pSM340) and *E. coli* Top10(pSM340) was enumerated by counting the GFP-expressing colonies that grew on Lennox broth–ampicillin agar.

**Quantitative PCR assay**—To determine the amount of *Salmonella* spp, a quantitative real-time PCR assay was used to enumerate the theoretic number of copies of sipB/C in the fecal samples and intestinal.
lumen contents. Fecal samples and luminal contents were processed to isolate genomic DNA by use of a commercially available kit in accordance with the manufacturer's instructions. Concentration of genomic DNA was determined by use of a spectrophotometer. Quantitative real-time PCR assay was performed as described elsewhere with sipB/C primers and a TaqMan probe in a total volume of 25 µL that contained 24 ng of genomic DNA. In addition, recovered Salmonella organisms were qualitatively assessed by the use of antibiograms and BJA8-specific primers (Tn5 and sipC-R).

**Figure 1**—Mean ± SEM in vitro anti-Salmonella activity of Escherichia coli P8E5. Lennox broth was coincubated on day 0 with Salmonella enterica serovar Typhimurium 968G and E. coli P8E5 (squares) or Salmonella Typhimurium 968G and E. coli Top10 (control sample; circles). Values reported represent the results for 5 separate experiments. *Value differs significantly (P < 0.05) from the value for the control sample.

PCR-based assessment of E. coli virulence genes in E. coli P8E5—To detect the virulence genes sia, sbi, stx1, stx2, stx2c, and eae, colonies of E. coli P8E5 were heated at 100°C for 10 minutes. Recovered DNA was subjected to quantitative real-time PCR assay as described previously.

**Antibiogram**—The antibiogram of E. coli P8E5 was determined in accordance with standards established by the Clinical and Laboratory Standards Institute. Assays were performed by use of E. coli P8E5 cultures in media that contained the appropriate antimicrobial at a concentration equivalent to its breakpoint, with aerobic growth overnight at 37°C.

**Effect of pH, proteolytic enzymes, heat, and storage on anti-Salmonella activity of E. coli P8E5 supernatant**—To test the effect of heat on anti-Salmonella activity, filter-sterilized E. coli P8E5 supernatants were heated at 60°C or 100°C for 30 minutes or autoclaved for 15 minutes. For the assessment of proteolysis, 50 µg of proteolytic enzyme was added to 50 µL of E. coli P8E5 supernatant, and the mixture was incubated at 37°C for 1 hour. To determine the effect of pH, filtered supernatants of E. coli P8E5 were adjusted to pH 4.0, 7.0, or 9.0 by the use of 1M HCl or 3M NaOH. Supernatants were also stored at 4º or –20°C for 2 weeks to 5 months to determine anti-Salmonella activity. Anti-Salmonella activity was evaluated by combining 100 µL of treated supernatants with 100-µL aliquots of Salmonella Typhimurium 968G (approx 2.5 X 10⁸ CFUs) in 96-well plates, which were incubated overnight at 37°C with shaking at 200 revolutions/min. Anti-Salmonella activity was visually determined by the lack of growth of Salmonella Typhimurium 968G (apparent OD₆₀₀ = 0) in the 96-well plate.

**Preliminary survey of the herd for E. coli P8E5**—Twenty additional fecal samples (10 from Salmonella-negative cattle and 10 from Salmonella-positive cattle) were obtained from the herd in Minnesota. Aliquots (2 g) of each fecal sample were inoculated into 50 mL of Lennox broth supplemented with P8E5-selective antimicrobials (ampicillin, 50 µg/mL; tetracycline, 16 µg/mL); solutions were incubated aerobically overnight at 37°C. Supernatants were collected after centrifugation at 1,500 X g for 15 minutes. Aliquots of bacteria-free supernatants were added to 96-well plates and coincubated with Salmonella Typhimurium—
um 968G as described previously. The 96-well plates were incubated overnight at 37°C with shaking at 200 revolutions/min. Fecal samples were designated as anti-Salmonella when growth of Salmonella organisms was not visually detectable (apparent OD₇₅₀ = 0) in 3 or more wells in the corresponding 96-well plate.

Statistical analysis—Significant differences between groups were identified by use of an ANOVA with the Scheffe F test for multiple comparisons. Significance was designated at values of P < 0.05.

Results

Bacterial identification and initial characterization—Supernatants of 19 fecal samples were initially tested for anti-Salmonella activity, and 1 of the 19 samples inhibited growth of Salmonella organisms. This fecal sample was obtained from a cow with no history of shedding Salmonella organisms. The bacterium with anti-Salmonella activity was isolated, identified as an E coli, and subsequently designated as P8E5. The antibiogram of E coli P8E5 included resistance to ampicillin, chloramphenicol, kanamycin, sulfamethoxazole, streptomycin, and tetracycline. The PCR analysis revealed that this strain did not possess virulence genes associated with production of Shiga toxin (stx1 or stx2), attachment and effacement (eca), enterotoxigenicity (sta or stb), or porcine edema disease (stx2c; data not shown). Anti-Salmonella activity was not detected in an additional 20 fecal samples from Salmonella-negative or Salmonella-positive cattle from the same herd (data not shown).

In vitro assessment of anti-Salmonella activity of E coli P8E5—Salmonella Typhimurium 968G was chosen for initial in vitro studies because its multiresistance phenotype could be exploited to ensure that E coli P8E5 supernatants were free of bacteria (ie, the addition of antimicrobials would ensure that the bacterial growth was Salmonella Typhimurium 968G and not E coli P8E5 that had contaminated the supernatant). Salmonella Typhimurium 968G was then used in the in vitro coculture experiments.

Significant inhibition of Salmonella Typhimurium 968G was detected when cultured in the presence of E coli P8E5 (Figure 1). After 24 hours of coculture, there was a 100-fold reduction in the recovery of Salmonella Typhimurium 968G, compared with the recovery for the E coli P8E5-free control sample. The magnitude of reduction in the recovery of Salmonella Typhimurium 968G continued to increase throughout the duration of the experiment. A 10,000-fold reduction was detected during the last week of the experiment. On the other hand, E coli P8E5 had a typical growth curve throughout the 8-day incubation period (data not shown).

In vivo assessment of anti-Salmonella activity of E coli P8E5—The ability of E coli P8E5 and E coli Top10 (control...
sample) to exclude Salmonella Typhimurium BJ68 was assessed in Holstein calves. Salmonella Typhimurium BJ68 was chosen for use in these experiments because it does not cause diarrhea or systemic disease, yet in our experience, we have found that it colonizes the bovine intestines. In the first in vivo experiment, Holstein calves were inoculated with Salmonella Typhimurium BJ68, evaluated for shedding of Salmonella organisms 3 days later, and then challenge exposed to E coli P8E5 or E coli Top10 (control sample). Quantitative real-time PCR assay revealed a 1,000-fold decrease, relative to the control sample, in shedding of Salmonella Typhimurium BJ68 in the feces of calves that were challenged exposed to E coli P8E5 (Figure 2). After day 15, shedding of Salmonella organisms ceased in the calves coinfected with Salmonella Typhimurium BJ68 and E coli P8E5, whereas shedding persisted in calves coinfected with Salmonella Typhimurium BJ68 and E coli Top10. Even though fecal shedding ceased, Salmonella Typhimurium BJ68 was detected in the luminal contents. However, a significant decrease in Salmonella Typhimurium BJ68 colonization was detected in the calves challenge exposed to E coli P8E5, compared with results for the control calves (Figure 3).

The second in vivo experiment involved inoculating Holstein calves with E coli Top10 or E coli P8E5 followed by inoculation with Salmonella Typhimurium BJ68 3 days later. There was more than a 10-fold difference in shedding of Salmonella Typhimurium BJ68 between the 2 test groups of calves on the third day after challenge exposure (Figure 4). At the end of the experiment, Salmonella Typhimurium BJ68 was reduced by approximately 10,000-fold in the calves inoculated with E coli P8E5. Salmonella Typhimurium BJ68 was detected in the luminal contents of the intestines from both groups of calves, with a 100-fold decrease in the E coli P8E5-treated calves (Figure 3). Colonization of E coli P8E5(pSM340) was evident in the calves throughout the experiment. However, E coli Top10(pSM340) was not detectable at 6 or more days after inoculation (data not shown).

In vitro assessment of the extent of the anti-Salmonella activity of E coli P8E5—To determine whether the bacteriocin-like activity extended to other microbes in addition to Salmonella Typhimurium, the supernatant of E coli P8E5 was tested against 2 S enterica serovars, including serovar Newport, which was identified in the endemic herd. There was a decrease in survival for the Salmonella serovars incubated with E coli P8E5 supernatant, compared with survival after incubation with the E coli Top10 supernatant (Figure 5). Furthermore, bacterial viability decreased for E coli Top10, E coli O157:H7, and Klebsiella pneumoniae exposed to the E coli P8E5 supernatant (mean ± SD reduction of 65.00 ± 3.70%, 99.97 ± 0.01%, and 80.10 ± 12.90%, respectively).

Characterization of bacteriocin-like activity—The bacteriocin-like activity of E coli P8E5 supernatants was detected at the temperatures tested (60° and 100°C for 30 minutes) but not after autoclaving. Bacteriocin-like activity was stable at pH 4, pH 7, and pH 9. Furthermore, this activity was still detectable after storage for 5 months at 4° and −20°C. The inhibitory activity was not detected after treatment with trypsin, chymotrypsin, and pronase. However, the activity was retained after treatment with pepsin or peptidase.

Discussion

In the study reported here, we determined anti-Salmonella effects of an indigenous E coli (ie, P8E5) cultured from a dairy herd with endemic Salmonella infection. Our original hypothesis was that a competitiveness exclusion bacterium maintains a Salmonella-free status, or at least minimizes shedding of Salmonella organisms, in a subpopulation of cattle within these types of herds. Of the 39 fecal samples tested for anti-Salmonella activity, 1 inhibited growth of Salmonella spp, although it is possible that other cattle in this herd harbored E coli P8E5. The single isolate suggests that the strain was unique to 1 animal or that our recovery process was not optimal for this strain.

In 2 separate experiments involving calves, E coli P8E5 was able to decrease shedding of Salmonella organisms. In these 2 experiments, the pathogen was administered before or after E coli P8E5 exposure. In both cases, Salmonella spp were not detected in the fecal samples at the end of the experiment, but they were detected in the luminal contents of the intestines. It
appears that E. coli P8E5 can minimize shedding but cannot completely eliminate colonization. In addition, E. coli P8E5 could only transiently colonize the calves in our experiments. Therefore, it is possible that E. coli P8E5 may have a cohort microbe that is required for stable colonization. Alternatively, E. coli P8E5 may have a greater affinity for adult versus neonatal animals.

Because the E. coli P8E5 anti-Salmonella activity was a protease-sensitive event, the basis for the anti-Salmonella effect is likely a bacteriocin or bacteriocin-like agent. Bacteriocins are proteins produced by bacteria for the purpose of inhibiting adjacent bacteria that compete for the same niche.10-13 These antimicrobial molecules are highly diverse and abundant, and they have been found in Eubacteria and Archaeabacteria.13,16 Bacteriocins are generally produced during stressful conditions and result in the rapid elimination of adjacent cells that are not immune or resistant to their effects.13,16 These antimicrobials are often plasmid encoded and contain genes for the bacteriocin, a lysis or export protein, and an immunity gene.12,15-20

*Escherichia coli* produces 2 types of bacteriocins, which are referred to as microcins and colicins.2,26,30 Microcins are smaller and can be chromosomally or plasmid encoded, and their synthesis is not lethal to the producing strain.7,12,31 Colicins are produced after the agonal-like response and are generally plasmid encoded.7 Colicin-producing communities have a rock-paper-scissors paradigm in which the colicin-producing bacteria (colicinogenic) instigate the formation of a community with 3 types of bacteria: colicinogenic, resistant, and sensitive.12,23 Sensitive bacteria predominate until a small fraction of the colicinogenic bacteria are lysed, release the colicin, and kill the sensitive bacteria.28 Resistant bacteria then supplant the colicinogenic bacteria because the latter must undergo autolysis for colicin release.28,29 Therefore, colicinogenic bacteria kill sensitive bacteria, which typically outcompete resistant bacteria and then outcompete colicinogenic bacteria, thus satisfying a rock-paper-scissors relationship.12,23,32,33 This paradigm illustrates how bacteriocins can influence population dynamics and biodiversity.28,32,33

To determine whether the bacteriocin-like activity of *E. coli* P8E5 is plasmid encoded, we cultured *E. coli* P8E5 in the presence of ethidium bromide as a plasmid-curing agent. This curing treatment reduced but did not abolish the activity of the supernatant (data not shown), which suggested that this treatment could eliminate this presumptive plasmid from only a fraction of the bacterial population in the treated culture. We were not able to isolate this plasmid by use of standard plasmid preparation methods. Thus, it appears that the bacteriocin-like activity is plasmid based, although other possible explanations may be valid.

In the study reported here, we isolated an *E. coli* that appeared to provide at least a partial homeostatic mechanism for assuaging salmonellosis in an endemically infected dairy herd. *Escherichia coli* P8E5 appeared to have bacteriocin-like properties against *Salmonella* spp, but the exact mechanism remains to be determined. Additionally, this strain may be able to do the same for *E. coli* O157:H7. *Escherichia coli* P8E5 illustrates the balance between pathogenic and avirulent intestinal microbiota, especially with regard to chronic problems associated with *Salmonella* infection. However, this particular strain represents only a tiny fraction of the complex gastrointestinal microbial community.

References

11. Stern NJ, Svetoch EA, Eruslanov BV, et al. Isolation of a *Lactobacillus salivarius* strain and purification of its bacteriocin, which is inhibitory to *Campylobacter jejuni* in the chick-
Appendix

Bacterial strains and plasmids used in the study to evaluate Salmonella infections in cattle.

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<thead>
<tr>
<th>Strain or plasmid</th>
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| *Salmonella enterica* | serovar Typhimurium 968G | Phage type DT104; resistance pattern Amp, Chl, Kan, Smx, Stp, Tet, and Zo 
| | serovar Typhimurium BJ68 | Strain SL1344; resistance pattern Tet 
| | serovar Newport | Cattle isolate, SARB 
| | serovar Dublin | Cattle isolate, SARB 
| | *Escherichia coli* P8E5 | Fecal isolate possessing anti-Salmonella activity; resistance pattern Amp, Chl, Kan, Smx, Stp, and Tet 
| | *E. coli* Top10 | F-mcrA Δ(mrr-hsRMS-mcrBC) g68lasZΔM15 ΔlacZΔΔlacI16lacF2ΔaraBAD10Δ araBAD16 lacI16 lacI16 lacI16 Δ(aarleu)7697 g3Δ K rpsL (StrR) endA1 nupG 
| Plasmid pSM340 | GFP-expressing plasmid; resistance pattern Amp; no effect on P8E5 activity 