Effects of microcin 24-producing *Escherichia coli* on shedding and multiple-antimicrobial resistance of *Salmonella enterica* serotype Typhimurium in pigs

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Objective—To investigate the effect of an *Escherichia coli* that produced microcin 24 (Mcc24) on shedding of *Salmonella enterica* serotype Typhimurium in swine and evaluate evidence of in vivo activation of the Mcc24-mediated, multiple-antibiotic resistance (*mar*) operon.

Animals—36 crossbred weaned pigs.

Procedure—24 pigs were allocated to 2 groups (12 pigs/group). Pigs in 1 group received daily oral administration of an Mcc24-producing *E coli*, whereas the other group received a non–Mcc24-producing *E coli*. All pigs were challenge exposed with *Salmonella* Typhimurium χ4232. A third group of 6 pigs received Mcc24-producing *E coli* and was challenge exposed with an Mcc24-sensitive, *marA*-deleted strain of *Salmonella* Typhimurium 4232. After challenge exposure, fecal samples from all pigs were cultured to detect shedding of *Salmonella* Typhimurium and *Salmonella* Typhimurium isolates were screened for resistance to ciprofloxacin. Fecal samples were collected throughout the study, and tissue samples were collected during necropsy.

Results—Differences in shedding of *Salmonella* Typhimurium were not detected between groups receiving Mcc24-producing or non–Mcc24-producing *E coli*. No significant differences were found in quantitative analysis between groups receiving Mcc24-producing and non–Mcc24-producing *E coli*. Evidence of *mar* activation was not detected.

Conclusions and Clinical Relevance—Microcin-producing *E coli* did not exert an effect on shedding of *Salmonella* Typhimurium or *mar* activation in pigs. It may be difficult or impractical to create the conditions required for Mcc24 to be an effective part of a food safety intervention to reduce shedding of *Salmonella* Typhimurium. (*Am J Vet Res* 2004;65:1616–1620)

Microcins (Mccs) are a group of antimicrobial peptides produced by Enterobacteriaceae that are active mainly against phylogenetically related bacteria.[1] They are believed to play an important role in the microbial ecosystem of the intestine.[2-4] **Microcin 24 (Mcc24)** has been isolated from a uropathogenic strain of *Escherichia coli* and can inhibit growth of *Salmonella* strains.[5]

Expression of Mcc24 in an avirulent strain of *E coli* isolated from birds led to the inhibition of shedding of *Salmonella enterica* serotype Typhimurium in intestinal tracts of chickens.[6] Additionally, Mcc24 from this same strain of *E coli* strongly inhibited strains of *Salmonella* organisms obtained from reptiles during an in vitro study.[7] On the basis of analysis of results of these studies, it has been suggested that Mcc24 may be used in a food safety intervention to exclude *Salmonella* spp and may provide an alternative to antimicrobial use.

In another study[8] conducted by our laboratory group, we reported that *Salmonella* Typhimurium could develop resistance to Mcc24 after repeated exposure in vitro. Additionally, we reported that acquisition of resistance to Mcc24 in *Salmonella* Typhimurium led to concurrent resistance to multiple antimicrobials (ciprofloxacin, tetracycline, chloramphenicol, and rifampin) through activation of the **multiple-antibiotic resistance (*mar*)** operon.[8] This operon regulates resistance to a number of foreign substances in *Salmonella* spp, principally through activation of a multiple-drug efflux pump[9,10]; the gene responsible for this activation is *marA*.[11]

The objectives of the study reported here were to determine whether an Mcc24-producing *E coli* fed daily to pigs could reduce shedding of *Salmonella* Typhimurium after challenge exposure and investigate the possibility that the *mar* system could be activated in *Salmonella* Typhimurium exposed to Mcc24 in vivo by testing for induction of resistance to ciprofloxacin.

Materials and Methods

Animals—Thirty-six 17-day-old crossbred pigs were used in the study. Approximately 30 days before the study, fecal samples were collected from the dams and screened by use of microbial culture for *Salmonella* organisms. Offspring were selected from sows that had negative results. Fecal sam-

Received February 27, 2004.
Accepted April 23, 2004.
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The authors thank H. K. Mahanty for providing the *Escherichia coli* pGOB18 and L. Zeller, Becky Ford, Jennifer Ohlfest, Gregory Phillips, and Tom Stabel for technical assistance.
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ples were collected from the pigs used in this study on days –7, –4, and 0 before challenge exposure with *Salmonella* Typhimurium and screened by use of microbial culture for *Salmonella* organisms.

Pigs were housed in pens (3 pigs/pen) with a solid concrete floor in an isolation barn at the National Animal Disease Center in Ames, Iowa. There was 1 pen/room. Pigs were fed a commercial ration that did not contain antimicrobials, and water was available ad libitum. All pigs were monitored daily to determine attitude, appetite, and consistency of feces. All pigs used in the study were handled and treated in compliance with a protocol certified by the National Animal Disease Center Institutional Animal Care and Use Committee protocol.

Bacterial strains and plasmids—All strains were stored at –70°C in 20% glycerol (vol:vol) prior to the start of the study. Genes responsible for Mcc24 production, immunity, and secretion were isolated from a 5.25-kb fragment of p24-2 harbored in *E coli* 2424.[a] The insert was subsequently cloned into pBR322 to produce the recombinant plasmid pGOB18.[a] Aliquots of *E coli* K-12 strain, MC4100,[12] were prepared and electroporated[b] (25 µF, 2.5 kV, 200 Ω) with 100 ng of pGOB18 or insertless pBR322,[13] thus generating *E coli* GOB18 and *E coli* BR322, respectively. *Escherichia coli* GOB18 has Mcc24-specific activity that inhibits the growth of *Salmonella* Typhimurium and *E coli* MC4100, whereas *E coli* pBR322 does not have similar activity. Additionally, insertion of the desired plasmid in *E coli* MC4100 was confirmed by use of a **polymerase chain reaction (PCR)** assay. Both *E coli* GOB18 and *E coli* BR322 strains were resistant to ampicillin as the result of a β-lactamase gene contained in the pBR322 plasmid. Plasmid stability was tested by serially passaging *E coli* GOB18 in Luria-Bertani broth without ampicillin in overnight cultures and testing for Mcc24 activity.

Nalidixic acid-resistant ***Salmonella* Typhimurium χ4232 (ST_{4232})**[14] and a modified **ST_{4232} with *marA* deletion (ST_{4232}Δ*marA*)** were used for challenge exposure. The ST_{4232}Δ*marA* was constructed in a manner similar to that reported elsewhere.[15] Briefly, primers were constructed that contained homologous sequences to DNA upstream and downstream of ST_{4232} *marA*. Each primer also had a sequence that allowed it to bind to the plasmid pKD3.[15] A PCR procedure was performed by use of pKD3 as a template; this yielded a linear piece of DNA that encoded resistance to chloramphenicol, which was flanked by a 50-bp *marA* sequence on each end. This linear fragment was transformed into ST_{4232} containing pKD46,[15] which encodes the genes needed for Red recombinase. Colonies resistant to chloramphenicol were selected, and the desired mutation was confirmed by use of a PCR technique.

The ST_{4232} and ST_{4232}Δ*marA* strains for challenge exposure were grown on blood agar plates, and colonies were transferred into prewarmed Luria-Bertani medium that contained nalidixic acid[c] (50 µg/mL). Cultures were incubated with shaking (220 revolutions/min)[d] for 3.5 hours at 37°C. Then, cultures were centrifuged (7,900 × *g* for 20 minutes), the supernatant removed, and the pellet resuspended in PBS solution to achieve the desired optical density (0.35 at a wavelength of 595 nm) and appropriate number of CFUs of *Salmonella* organisms per milliliter.

Mcc activity—Luria-Bertani plates were flooded with indicator strain (approx 2×10^8 of *E coli* MC4100, ST_{4232}, or ST_{4232}Δ*marA*) in broth and allowed to absorb the liquid for 2 to 5 minutes. Sterile blank disks[e] were placed on the plate surface and soaked with test strain (10 µL of *E coli* GOB18). Plates were incubated overnight at 37°C and examined for zones of inhibition. Alternatively, test strains were selected and a sterile toothpick was used to obtain material from test strains and inoculate it onto indicator-flooded plates.

Experimental design—Twenty-four pigs were allocated to 2 groups (12 pigs/group). Each day, pigs in group 1 were orally administered Mcc24-producing *E coli*, whereas pigs in group 2 received non–Mcc24-producing *E coli*. The oral administration of *E coli* began 4 days prior to challenge exposure with *Salmonella* Typhimurium (day –4). Daily oral administration of each strain consisted of 2 mL (approx 2×10^8 CFUs) of culture material, which was obtained after overnight culture in Luria-Bertani broth[f] containing ampicillin[g] (100 µg/mL). Each strain was tested daily for Mcc24 activity against *E coli* MC4100. All pigs in both of these groups were challenge exposed with ST_{4232} on day 0. A final plate count of the solution indicated a concentration of 2.0×10^8 CFUs of *Salmonella*/mL in the inoculum used for challenge exposure.

Pigs of group 3 (n = 6) were orally administered Mcc24-producing *E coli* and challenge exposed with the *marA*-deleted strain of *Salmonella* Typhimurium 4232 (ie, ST_{4232}Δ*marA*). In addition, 2 other groups of pigs (3 pigs/group) were not administered *E coli* but were challenge exposed with ST_{4232} (group 4) or ST_{4232}Δ*marA* (group 5). *Escherichia coli* administration in group 3 and *Salmonella* Typhimurium challenge exposure in groups 3, 4, and 5 were performed in parallel with groups 1 and 2.

Fecal samples were collected from each pig by use of a plastic sleeve[h] that attached to a thermometer probe, and samples from the pen floor were collected from a mixed sample of feces. On day 20 of the study, pigs were euthanized and necropsies were performed. Samples collected aseptically during necropsy included portions of the ileocecal lymph nodes, a segment (approx 10 cm) of the distal portion of the ileum, colon contents, and cecal contents.

Processing of samples—All samples were transported to our laboratory within 1 hour after collection. Samples of ileocecal lymph nodes and the distal portion of the ileum were collected aseptically, weighed, and placed in a sterile zipper-seal bag. Then, an amount of 0.1% **buffered peptone water (BPW)** equivalent to 2 times the weight of the sample was added to the bag. Samples were macerated with a rubber mallet, and each sample was homogenized by use of a stomacher[i] (260 rounds/min for 1 minute). One milliliter of the homogenate was added to 9 mL of BPW. Samples of colon and cecal contents were weighed and diluted 1:9 (wt:wt) in BPW. Weight of fecal samples was determined by subtracting the sleeve weight from the combined weight of the sleeve and fecal sample. Fecal samples were diluted 1:9 (wt:wt) in BPW. When the calculated amount of BPW for dilution was < 1.0 mL, the volume was adjusted to 1.0 mL. The initial 1:10 dilutions were serially diluted (one-tenth dilutions), and 10-µL aliquots of each dilution were streaked in triplicate onto **xylose-lysine-tergitol (XLT)** agar[j] that contained nalidixic acid (50 µg/mL); this agar was used because ST_{4232} is resistant to nalidixic acid. Additionally, 100 µL of the initial 1:10 dilution was spread onto XLT agar that contained nalidixic acid (50 µg/mL), XLT agar that contained nalidixic acid (50 µg/mL) and ciprofloxacin[k] (4 µg/mL), and MacConkey medium with ampicillin (100 µg/mL). All plates were incubated at 37°C for 24 hours. After incubation, suspected *Salmonella* colonies were enumerated and ampicillin-resistant colonies with typical *E coli* morphology were selected and tested for Mcc24 activity.

Enrichment medium was used to further evaluate *Salmonella* organisms. The samples in BPW were incubated at 37°C for 24 hours. An aliquot (100 µL) of the mixture was then inoculated into 10 mL of **Rappaport-Vassiliadis (RV)** medium[l] that contained nalidixic acid (50 µg/mL) and incubated at 37°C for 24 hours. An aliquot from this incubation was used for a second incubation in RV medium that contained nalidixic acid. Aliquots (100 µL) of the second incu-

bation in RV medium were spread onto XLT agar that contained nalidixic acid or XLT agar that contained nalidixic acid and ciprofloxacin; these plates were incubated at 37°C for 24 hours and observed for growth. Suspected *Salmonella* colonies were transferred onto Rambach[16] medium,[m] incubated at 37°C for 24 hours, and evaluated for the typical morphologic appearance and color of *Salmonella* colonies. Antimicrobial-resistance patterns of the test organisms were determined by use of the Kirby-Bauer method.[17] Antimicrobials used for testing included ampicillin, chloramphenicol, ciprofloxacin, rifampin, and tetracycline.

PCR amplification of the Mcc24 gene—A PCR assay was conducted to detect the **Mcc24 structural gene (*mtfS*)** in diluted fecal and tissue samples obtained from various pigs, in various samples, and on various days. Additionally, the PCR technique was conducted to confirm *mtfS* in selected bacterial colonies that had typical *E coli* morphology and potential Mcc24 activity in isolates grown on MacConkey medium[n] with ampicillin (100 µg/mL). Extraction of plasmid DNA was performed by use of a miniprep kit.[o] Forward and reverse primers (corresponding to sequences within *mtfS*) used were 5'–GCT GGA GAT CCG CTT GCA GAT–3' and 5'–TTA TCC TTT ACT TCC GTT CCA–3', respectively. The PCR amplification was performed by use of 0.2-mL tubes in an automated thermocycler.[p] Reactions were performed in a reaction volume of 20 µL that contained 200µM deoxyadenosine triphosphate, 200µM deoxythymidine triphosphate, 200µM deoxycytidine triphosphate, and 200µM deoxyguanosine triphosphate; 0.5µM of each primer; 0.5 units of *Taq* DNA polymerase[q]; 50 ng of template; 1X PCR buffer; and 5 µL of water. Thermocycling entailed 94°C for 3 minutes and then 35 cycles (94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 30 seconds). Visual detection of 225-bp amplicons was performed following electrophoresis on agarose gels.

Statistical analysis—The proportion of pigs that had positive results for culture of *Salmonella* organisms in enriched medium was assessed by use of a generalized linear model with a logit-link function.[18] Shedding of *Salmonella* organisms in pigs (culture results before use of enrichment media) was analyzed by use of a 1-way ANOVA to evaluate differences among days and tissues. Additionally, data for culture results before use of enrichment media were analyzed by use of a mixed linear model with effects for group, day of study, and group-by-day interaction. Also, Wilcoxon rank sum tests were conducted among groups for each tissue type, and the maximum bacterial count for each day was obtained. Effect of sample weight was considered in the analysis of data for culture results before and after use of enrichment media. A value of $P \leq 0.05$ was considered significant. All results were analyzed by use of a commercially available statistical software package.[r]

Results

Two pigs (1 from group 3 and 1 from group 5) had positive culture results for *Salmonella* spp prior to challenge exposure with *Salmonella* Typhimurium and were excluded from the study. All other pigs had negative culture results for *Salmonella* spp prior to challenge exposure with ST_{4232} or $ST_{4232}\Delta marA$.

We detected Mcc24 production by *E coli* GOB18 after 20 consecutive passages in Luria-Bertani broth without ampicillin and after 20 days in sterile water at 25°C. *Escherichia coli* that produced Mcc24 was isolated from the feces of pigs in groups 1 and 3 (7/728 [< 1.0%] isolates tested). Fecal or tissue samples from pigs in groups 1 and 3 had positive results (11/96 [11.4%]) when tested by use of the PCR assay for the *mtfS* gene (data not shown).

Culture results by use of enrichment medium for samples obtained from pigs that received ST_{4232} and *E coli* GOB18 or *E coli* BR322 were summarized (**Figure 1**). The percentage of pigs that had positive results for culture of *Salmonella* organisms did not differ significantly between the group that received Mcc24-producing *E coli* and the group that did not receive Mcc24-producing *E coli*. *Salmonella* spp was detected in a high percentage (≥ 75%) in fecal samples of groups 1 and 2 collected throughout the study and tissue samples collected during necropsy. Weight of each fecal sample was considered as a covariate but did not provide an important contribution to the predictive capability of the model; therefore, it was removed from the final model.

Results of culture without enrichment medium for samples obtained from pigs that received ST_{4232} and Mcc24-producing or non–Mcc24-producing *E coli* were summarized (**Figure 2**). Analysis of these results indicated a lower number of *Salmonella* spp recovered in fecal samples during the first 10 days following challenge exposure and from ileocecal lymph nodes obtained during necropsy (day 20) from pigs that received Mcc24-producing *E coli*. However, there were no significant differences among groups on a day- or tissue-basis, as determined by use of the 1-way ANOVA. We did not detect significant differences by use of the mixed linear model or Wilcoxon rank sum test between groups that received Mcc24-producing and non–Mcc24-producing *E coli*. Again, weight of the fecal sample did not prove to be an important factor in the predictive capability of the models.

Pigs had a mean increase in rectal temperature of 0.53°C for the first 4 days after challenge exposure with

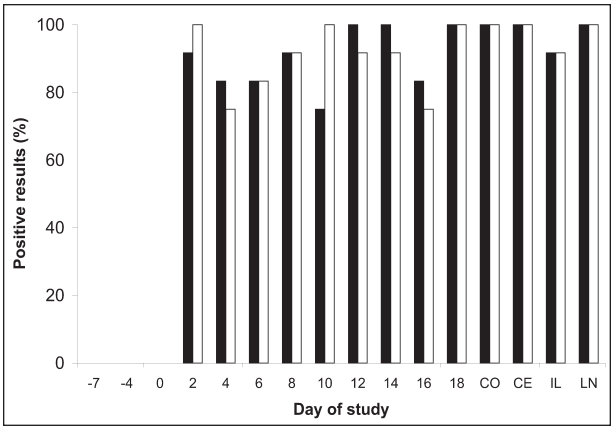

Figure 1—Percentage of pigs that received daily oral administrations of *Escherichia coli* GOB18 (microcin [Mcc]-producing *E coli*; black bars) or *E coli* BR322 (non–Mcc-producing *E coli*; white bars) followed by challenge exposure with *Salmonella enterica* serotype Typhimurium and shed *Salmonella* Typhimurium in feces and tissue samples. There were 12 pigs in each group. The first day of oral administration of *E coli* was on day –4. The day of challenge exposure with *Salmonella* Typhimurium was day 0. Results are expressed as the percentage of pigs with positive results for culture by use of enrichment medium. Fecal samples were collected on days –7, –4, and 0 and every other day after challenge exposure for 20 days. Tissue samples were collected from the colon (CO), cecum (CE), ileum (IL) and lymph nodes (LN) during necropsy performed on day 20.

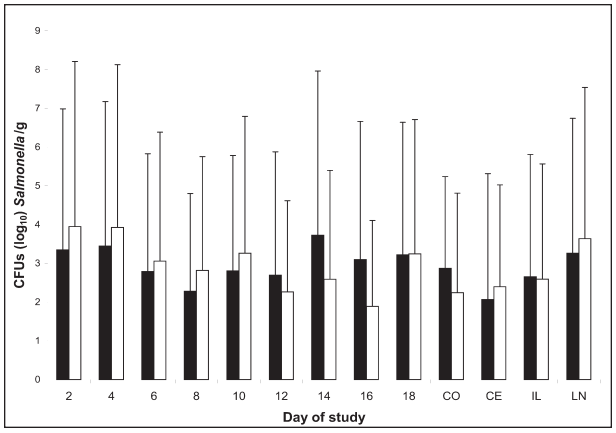

Figure 2—Quantitative analysis of *Salmonella* Typhimurium isolated by use of enrichment medium from feces and tissue samples obtained from pigs that received daily oral administrations of Mcc24-producing *E coli* (black bars) or non–Mcc24-producing *E coli* (white bars). Results were expressed as the mean + SD log_{10} number of CFUs of *Salmonella* organisms per gram of sample for each group. *See* Figure 1 for remainder of key.

Salmonella Typhimurium, compared with rectal temperatures before challenge exposure. Most pigs had a moderate decrease in appetite, were moderately lethargic, and had loose feces for 1 to 3 days following challenge exposure with *Salmonella* Typhimurium; however, we did not detect significant clinical differences (rectal temperature, appetite, attitude, or fecal consistency) between groups that received Mcc24-producing *E coli* and non–Mcc24-producing *E coli* (data not shown). Similarly, we did not detect significant differences in shedding of *Salmonella* organisms or clinical signs between pigs in the groups that received only ST_{4232} or $ST_{4232}\Delta marA$ and the remainder of the pigs (data not shown).

Analysis of antimicrobial resistance revealed no change in the antibiograms of ST_{4232} isolated from fecal samples obtained from various pigs or pen floors. Specifically, the nalidixic acid-resistance phenotype was retained, but resistance was not observed for ampicillin, chloramphenicol, ciprofloxacin, rifampin, or tetracycline. Additionally, resistance to Mcc24 was not observed.

Discussion

In other studies, Mcc24 caused decreased shedding of *Salmonella* Typhimurium from chickens in vivo[6] and inhibited growth of *Salmonella* Typhimurium isolates from reptiles in vitro.[7] However, another in vitro study[8] revealed that Mcc24 can induce a multiple-drug resistance phenotype in *Salmonella* Typhimurium through activation of the *mar* operon. This includes acquired resistance to ciprofloxacin, an important antimicrobial used to treat infections in humans. For the conditions of the study reported here, Mcc24-producing *E coli* did not decrease shedding of *Salmonella* Typhimurium in pigs after challenge exposure or induce resistance to ciprofloxacin through *mar* activation. Although an apparent reduction in *Salmonella* Typhimurium shedding attributable to the effects of Mcc24-producing *E coli* in the first 10 days following challenge exposure, in addition to a reduction in invasion of the ileocecal lymph nodes, may have been evident, it was not supported by results of statistical analysis. There was no difference in the percentage of pigs that shed *Salmonella* Typhimurium, regardless of whether they were fed Mcc24-producing *E coli*. Results of the study reported here do not eliminate the possibility that Mcc24-producing *E coli* could be effective in excluding *Salmonella* Typhimurium; however, they do indicate that it may be difficult or impractical to achieve the conditions necessary for this to happen.

Consideration of the bacterial host is an obvious factor. We chose to use a non–host-adapted strain of *E coli* that efficiently expressed Mcc24. In this manner, we could assess the effect of transient passage of an Mcc24-producing *E coli* in the gastrointestinal tract on unattached *Salmonella* organisms. Colonization in the intestines with established microflora is a highly variable event; assuming an effect was detected, it would be difficult to determine whether establishment of microflora was a necessary requirement. It is worthy of mention that even when the Mcc24-producing bacterium was a wild-type strain, it did not colonize 1-day-old chicks.[6] The possibility exists that our host failed to survive in adequate numbers for Mcc24 to affect shedding of *Salmonella* Typhimurium, even though we were able to isolate Mcc24-producing *E coli* from the feces of pigs that received it orally.

Subsequent studies should consider characterization of swine-adapted strains of *E coli* for intestinal survival, colonization, and Mcc24 production in an attempt to increase the amount of Mcc24 in the gastrointestinal tract. Although Mccs are believed to remain active in the intestines,[2,19] Mcc24 appeared less active when there were cell-free intestinal or fecal liquids in the study reported here (data not shown). Possible in vivo inhibition of Mcc24 could be further characterized by conducting bioreactor experiments, as has been reported for MccJ25.[19]

Additionally, the use of gnotobiotic pigs, rather than conventionally raised pigs, would simplify the intestinal environment and provide a clearer picture of the in vivo activity of Mcc24 against *Salmonella* Typhimurium. It would also more closely approximate the conditions of another study[6] that involved the use of 1-day-old chicks that would not be expected to have an established intestinal microflora.

The method for delivery of Mcc24-producing *E coli* should also be considered in subsequent studies. Even though we orally inoculated pigs with a high dose on a daily basis, a more continuous exposure to Mcc24 may be needed to be effective. Other investigators[6] were able to document an effect when Mcc24-producing *E coli* were delivered continually in the drinking water but not when administered orally prior to challenge exposure.

It may be possible to produce Mcc24 from *Salmonella* Typhimurium, which opens the possibility for the use of a nonpathogenic *Salmonella* Typhimurium host for delivery. This would likely increase the amount of Mcc24 in the *Salmonella* Typhimurium microenvironment but may also raise public health considerations. Another consideration is

heterologous expression of Mcc24 from a potentially beneficial intestinal microorganism, such as *Bifidobacteria* sp. However, an attempt by our laboratory group to express Mcc24 from lactic acid bacteria by use of methods described elsewhere[20] has not been successful (data not shown).

We did not find evidence of *mar* activation of *Salmonella* Typhimurium. This has been reported[8] in vitro after repeated exposure of *Salmonella* Typhimurium to the highest dose of Mcc24 tolerable. Intestinal conditions may not allow the amount of Mcc24 exposure achievable in vitro to be duplicated in vivo; however, increasing the amount of Mcc24-producing *E coli* in the gastrointestinal tract or more closely matching the Mcc24-producing *E coli* with the *Salmonella* microbiological niche may lead to *mar* activation.

Analysis of results of the study reported here indicates that the use of an Mcc24-producing *E coli* to reduce the number of *Salmonella* Typhimurium in the intestinal contents and feces of pigs will require detailed knowledge of the intestinal microbiological conditions that are conducive to competitive exclusion. Although Mcc24 does have inhibitory activity against various *Salmonella* serotypes in vitro, extrapolation to a complex ecosystem such as the gastrointestinal tract may prove daunting. The additional concerns of resistance and cross-resistance to antimicrobials also require additional evaluation. Given all of these complexities and concerns, it appears unlikely that Mcc24-producing *E coli* will be a paradigm for the use of Mcc-based probiotics in swine.

[a] O'Brien G. *Molecular analysis of microcin 24: genetics, secretion, and mode of action of a novel microcin*. PhD dissertation, Department of Cellular and Molecular Biology, University of Canterbury, 1996.
[b] Gene-Pulser II, BioRad Laboratories, Hercules, Calif.
[c] Nalidixic acid, Sigma Chemical Co, St Louis, Mo.
[d] Incubated Shaker Model C-24, New Brunswich Scientific, Edison, NJ.
[e] Bacto concentration discs, Becton-Dickinson, Sparks, Md.
[f] Luria-Bertani broth, Becton-Dickinson, Sparks, Md.
[g] Ampicillin, Sigma Chemical Co, St Louis, Mo.
[h] Plastic sleeves, Relion, Omron Healthcare Inc, Vernon Hills, Ill.
[i] Stomacher 400 circulator, Seward Ltd, London, UK.
[j] XLT 4 agar, Becton-Dickinson, Sparks, Md.
[k] Ciprofloxacin, Sigma Chemical Co, St Louis, Mo.
[l] Rappaport-Vassilialis media, Becton-Dickinson, Sparks, Md.
[m] Rambach media, Chromagar Microbiology, Paris, France.
[n] MacConkey agar, Becton-Dickinson, Sparks, Md.
[o] Eppendorf Perfectprep, Brinkman Instruments, Westbury, NY.
[p] Thermocycler, Hybaid, Teddington, UK.
[q] *Taq* DNA polymerase, QIAGEN Inc, Valencia, Calif.
[r] SAS/STAT, version 8, SAS Institute Inc, Cary, NC.

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