Selection of Fecal Enterococci Exhibiting *tcrB*-Mediated Copper Resistance in Pigs Fed Diets Supplemented with Copper[∇][†]

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Copper, as copper sulfate, is increasingly used as an alternative to in-feed antibiotics for growth promotion in weaned piglets. Acquired copper resistance, conferred by a plasmid-borne, transferable copper resistance (*tcrB*) gene, has been reported in *Enterococcus faecium* and *E. faecalis*. A longitudinal field study was undertaken to determine the relationship between copper supplementation and the prevalence of *tcrB*-positive enterococci in piglets. The study was done with weaned piglets, housed in 10 pens with 6 piglets per pen, fed diets supplemented with a normal (16.5 ppm; control) or an elevated (125 ppm) level of copper. Fecal samples were randomly collected from three piglets per pen on days 0, 14, 28, and 42 and plated on M-*Enterococcus* agar, and three enterococcal isolates were obtained from each sample. The overall prevalence of *tcrB*-positive enterococci was 21.1% (38/180) in piglets fed elevated copper and 2.8% (5/180) in the control. Among the 43 *tcrB*-positive isolates, 35 were *E. faecium* and 8 were *E. faecalis*. The mean MICs of copper for *tcrB*-negative and *tcrB*-positive enterococci were 6.2 and 22.2 mM, respectively. The restriction digestion of the genomic DNA of *E. faecium* or *E. faecalis* with S1 nuclease yielded a band of ~194-kbp size to which both *tcrB* and the *erm*(B) gene probes hybridized. A conjugation assay demonstrated cotransfer of *tcrB* and *erm*(B) genes between *E. faecium* and *E. faecalis* strains. The higher prevalence of *tcrB*-positive enterococci in piglets fed elevated copper compared to that in piglets fed normal copper suggests that supplementation of copper in swine diets selected for resistance.

Copper is an essential trace element required for vital biological functions in both prokaryotic and eukaryotic cells (29). The growth response in piglets to copper is independent of—and in addition to—the response to commonly used antibiotics in the feed (16). Copper in excess concentrations is toxic to cells because it induces the production of intracellular reactive oxygen radicals, which inactivate cell components such as nucleic acids, lipids, and proteins (32). Therefore, cells tightly regulate intracellular copper concentrations to avoid toxicity (43). The copper homeostasis mechanism has been well studied in certain Gram-positive bacteria, such as *Enterococcus hirae*, *Lactococcus lactis*, and *Bacillus subtilis* (45). The normal intracellular copper concentration is maintained by the *copYZAB* operon, where *copA* and *copB* encode copper transport ATPases responsible for the influx and efflux of copper, respectively. The *copY* gene acts as a copper-responsive repressor and *copZ* encodes a copper chaperone (46).

Some bacteria acquire resistance to copper, which may be either chromosome mediated (17) or plasmid mediated (31). A plasmid-borne gene, designated as transferable copper resistance (*tcrB*) and homologous to *copB* of the *copYZAB* operon, was reported in *E. faecium* and *E. faecalis* isolated from piglets, calves, poultry, and humans in Denmark (2). In Denmark, the plasmid also carries the genes *erm*(B) and *vanA*, which encode resistance to macrolides and glycopeptides, respectively (2, 20), suggesting a potential link between copper resistance and antibiotic resistance. We have confirmed the occurrence of the *tcrB* gene in fecal enterococci of piglets fed diets with normal or elevated levels of copper (4). Whether copper supplementation in pigs exerts selection pressure for copper-resistant enterococci, which in turn could lead to coselection for macrolide or glycopeptide resistance, has not been documented. Because of the limited number of isolates tested in our previous study, we were unable to relate copper supplementation to the prevalence of *tcrB*-positive enterococci. Therefore, the present longitudinal study was undertaken to determine the relationship between supplementation of copper at an elevated concentration (125 ppm) and the prevalence of fecal *tcrB*-mediated copper-resistant and *erm*(B)-mediated macrolide-resistant enterococci in piglets. We performed Southern blot hybridization to show that the *tcrB* gene is carried on a transferable plasmid and provide evidence for the interspecies cotransfer of *tcrB* and *erm*(B) genes in enterococci.

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MATERIALS AND METHODS

Animals, experimental design, and sampling. The use of animals and the experimental procedure followed were approved by the Kansas State University Animal Care and Use Committee. Sixty newly weaned piglets (21 days old) with an average body weight of 7.0 kg (± 1 kg) were randomly allocated to two dietary

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treatments. The two dietary treatments were as follows: basal diet with 16.5 ppm of copper (control group) or basal diet supplemented with 125 ppm of copper as copper sulfate (copper group). The basal diet consisted of corn, soybean meal, vitamins, amino acids, and trace mineral supplements, and piglets were housed in an environmentally controlled nursery facility. Each treatment group had a total of 30 piglets assigned to 5 pens with 6 piglets per pen. Each pen was provided with a self-feeder containing four holes and a water nipple such that animals had *ad libitum* access to feed and water. Each pen had a wire-mesh floor that allowed for 0.3 m² per piglet. Piglets were fed treatment diets for 42 days. Fecal samples were collected randomly from 3 pigs in each pen on days 0, 14, 28, and 42, placed in individual bags, and transported to the laboratory on ice.

Isolation of enterococci. Unless otherwise mentioned, all culture media used were from Difco (Becton Dickinson, Sparks, MD). Fecal samples were processed by diluting 1 g of feces in 10 ml of phosphate-buffered saline and spread plating 50 μl of the suspension onto M-*Enterococcus* agar. The plates were incubated for 24 h at 37°C. Five colonies (pinpoint red, pink, or metallic pink) were picked, streaked onto blood agar plates, and incubated overnight at 37°C. All isolates were tested for esculin hydrolysis by inoculating them into 100 μl of Enterococcosel broth in a 96-well microtiter plate (Becton Dickinson, Franklin Lakes, NJ) and incubating at 37°C for 4 h. Three esculin hydrolysis-positive isolates per fecal sample (9 per pen and 45 per treatment at each sampling time) were stored using Protect beads (Cryocare; Key Scientific Products, Stamford, TX) at −80°C until further use.

PCR for detection of the *tcrB* gene. The *tcrB* gene was detected according to the procedure described by Hasman et al. (20). Each isolate from a single Protect bead was streaked onto a blood agar plate and incubated overnight at 37°C. Bacterial DNA was extracted by suspending a single colony in nuclease-free water with Chelex 100 Resin (Bio-Rad Laboratories, Hercules, CA) and boiling it for 10 min (4). A *tcrB*-positive *E. faecium* strain (7430275-4 or 7430272-6; kindly provided by Henrik Hasman, National Food Institute, Technical University of Denmark) served as the positive control.

Speciation of *tcrB*-positive enterococci. Species identification of the enterococcal isolates that were *tcrB* positive and an equal number of *tcrB*-negative isolates, randomly chosen from the control and treatment groups, was performed by a multiplex PCR that identifies *E. faecium*, *E. faecalis*, *E. gallinarum*, and *E. casseliflavus* (24). Additionally, superoxide dismutase (*sodA*) gene sequence analysis (42) was used for species confirmation. The DNA template was prepared as mentioned before. The ATCC (Manassas, VA) strains of *E. faecium* (ATCC 19434), *E. faecalis* (ATCC 19433), *E. gallinarum* (ATCC 49579), and *E. casseliflavus* (ATCC 25788) served as positive controls. Master mixes, primers, and running conditions for the multiplex PCR and *sodA* gene PCR were as described by Jackson et al. (24) and Poyart et al. (42), respectively. The *sodA* gene products were purified by the Wizard SV gel and PCR cleanup system (Promega, Madison, WI). The purified PCR products were sequenced at Genomics Core, Institute for Integrative Genome Biology, University of California at Riverside. The sequences were analyzed by a BLAST search in the NCBI GenBank database.

Detection of *erm*(B) and *vanA* genes. The primers and PCR conditions for detection of *erm*(B) and *vanA* genes were as per the work of Jacob et al. (26) and Kariyama et al. (27), respectively. *Enterococcus faecalis* MMH 594 (provided by Lynn Hancock, Division of Biology, Kansas State University) and *E. faecium* (ATCC 51559) served as positive controls for the *erm*(B) and *vanA* genes, respectively.

Copper susceptibility determinations. Copper susceptibility determination for *tcrB*-positive and an equal number of *tcrB*-negative enterococcal isolates was performed by the agar dilution method (19, 20). The two strains from Denmark (7430162-6 and 7430275-4) were included as positive controls. Mueller-Hinton (MH) agar plates prepared with concentrations of copper, added as copper sulfate (Fischer Scientific, Fair Lawn, NJ), at 0, 2, 4, 8, 12, 16, 20, 24, 28, 32, 36, or 40 mM and with pH adjusted to 7.0 were used. The plates were spot inoculated with 20 μl of bacterial culture that was adjusted to McFarland turbidity standard no. 0.5 (Remel, Lenexa, KS) and incubated at 37°C for 48 h to determine growth or lack of growth. The susceptibility determinations were repeated on another day with different inoculum preparations.

Antibiotic susceptibility determinations. The microbroth dilution method was used to determine the MICs for erythromycin and vancomycin (Sigma-Aldrich, St. Louis, MO) per CLSI guidelines (11). Antibiotic stock solutions, each containing a final concentration of 1,000 μg/ml based on potency, were prepared in sterile distilled water. The bacterial cultures were prepared by inoculating a single colony in 10 ml MH broth, incubating for 6 h, and then diluting 100-fold with sterile MH broth. Antibiotics were tested at concentrations of 100, 50, 25, 12.5, 6.25, 3.125, 1.56, 0.78, 0.39, 0.195, and 0.098 μg/ml. The antimicrobial susceptibility test was performed in 96-well microtiter plates (Becton Dickinson), and inoculated plates were incubated at 37°C for 24 h, and results were recorded

as growth or no growth. The MIC determinations were repeated on another day with different inoculum preparations.

Pulsed-field gel electrophoresis. The pulsed-field gel electrophoresis (PFGE) analysis of *tcrB*-positive isolates was performed as per the work of Murray et al. (36) with minor modifications. A strain of *Salmonella enterica* serotype Braenderup H9812 was used as the standard (B. Murray, University of Texas Medical School, personal communication). A single colony of the isolate was inoculated into 5 ml of brain heart infusion (BHI) broth and incubated overnight at 37°C. One milliliter of overnight culture was transferred into a 1.5-ml Eppendorf tube, and cells were pelleted by centrifugation at 9,300 × *g* for 1 min. The supernatant was discarded and the pellet was suspended in 200 μl of 0.85% NaCl. The plugs were prepared by mixing 200 μl of the cell suspension with 200 μl of 1.6% SeaKem gold agarose. The plugs were lysed by transferring them into a 10-ml lysis solution (6 mM Tris-HCl, pH 7.4, 100 mM EDTA, 1 M NaCl, 0.5% sodium lauroyl sarcosine, 0.5% Brij, 0.2% deoxycholate, lysozyme [500 μg/ml] and RNaseA [20 μg/ml]) for 4 h at 37°C with gentle shaking. The plugs were then transferred to ESP buffer (10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1% SDS, and 50 μg/ml proteinase K) and incubated overnight at 50°C with gentle shaking. The plugs were washed three times at room temperature for 10 min each with TE dilute (10 mM Tris-HCl, pH-7.4, 0.1 mM EDTA) and stored at 4°C until used.

Restriction digestion of the plugs was performed by placing a small slice of agarose plug in a 1.5-ml microcentrifuge tube with 2 μl of SmaI in 10 μl of 10× buffer; the volume was increased to 100 μl with double distilled water and the mixture was held for 4 h at 25°C. The digested plugs were transferred onto a gel (1% SeaKem Gold Agarose with 0.5× Tris-borate-EDTA [TBE]) and the gel was placed in an electrical field device, CHEF-DR II (Bio-Rad, Richmond, CA), at 200 V. Pulse times were as follows: for block 1, an initial time of 3.5 s and a final time of 25 s for 12 h; and for block 2, an initial time of 1 s and a final time of 5 s for 8 h. Then the gel was stained with 0.0001% (or 1 μg/ml) ethidium bromide for 30 min followed by destaining in distilled water 3 times for 20 min each. Gel images were captured using a Gel Doc 2000 system (Bio-Rad), and band patterns were analyzed and compared by using BioNumerics version 3.0 (Applied Maths, Austin, TX). For clustering with a position tolerance setting of 1.5% for optimization and a position tolerance of 1.5% for band comparison, we used the band-based Dice similarity coefficient and the unweighted-pair group mathematical average algorithm method (unweighted-pair group method using average linkages [UPGMA]). Isolates were grouped based on identical banding patterns (100% Dice similarity).

Southern blot hybridization of *tcrB* and *erm*(B) genes. The Southern blot hybridization was performed on two *tcrB*-positive *E. faecium* isolates, one *tcrB*-positive *E. faecalis* isolate, and an equal number of *tcrB*-negative isolates by using digoxigenin (DIG) High Prime labeling and detection starter kit II (Roche Diagnostics, Indianapolis, IN). The procedure was performed with slight modifications to the instructions provided in the commercial kit. The genomic DNA was used to prepare plugs as in the PFGE protocol, which was followed by restriction digestion with S1 nuclease enzyme (New England BioLabs Inc., Beverly, MA). The restriction digestions were performed using 10 U of enzyme in 25 μl of buffer (44). The digested DNA samples were subjected to electrophoresis using the CHEF-DRII apparatus with Midrange II PFG molecular marker (New England BioLabs, Inc.). The staining and destaining procedures were carried out as in the PFGE protocol. After PFGE, each band was considered a unit length of linear plasmid. The gel was then subjected to depurination (0.25 N HCl), denaturation (1.5 M NaCl, 0.5 N NaOH), and neutralization (Tris base, NaCl, pH 7.4) buffers. The gel containing linearized and separated plasmid DNA was transferred onto a positively charged nylon membrane (Genescreen Plus, Boston, MA) and left overnight for blotting at room temperature by capillary transfer with 10× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) transfer buffer (pH 7.0). After blotting, DNA was immobilized by UV cross-linking for 2 min (UV Cross-linker, FB-UVXL-1000). The *tcrB* and *erm*(B) probes were prepared by random primer labeling using DIG High Prime. The immobilized DNA on the membrane was subjected to prehybridization using DIG Easy Hyb solution followed by hybridization with DIG-labeled DNA probe. The hybridization was carried out overnight at 37°C with gentle agitation. After hybridization, the membrane was developed using CL-XPosure X-ray film (Thermo Scientific, Rockford, IL).

Interspecies transferability of *tcrB* gene. A conjugation assay was performed using the filter mating procedure (47). The *tcrB*-positive donor strains (5 *E. faecium* and 5 *E. faecalis* strains) were resistant to tetracycline [MIC > 100 μg/ml; *tet*(M) positive] and susceptible to spectinomycin [MIC = 12.5 μg/ml]. *E. faecium* strain TX 5034 (provided by Barbara Murray, University of Texas Medical School), resistant to spectinomycin (MIC > 100 μg/ml) and *tet*(M) negative and susceptible to tetracycline (MIC = 0.78 μg/ml), and *E. faecalis* strain OG1SSp (provided by Ludek Zurek, Kansas State University), resistant to spec-

TABLE 1. Prevalence of *tcrB*-positive fecal enterococci in piglets fed diets supplemented with or without copper

Treatment (copper level in the feed)	No. of isolates positive for *tcrB* gene/total isolates tested				
	Sampling day				Total (%)
	0	14	28	42	
Control (16 ppm)	0/45	3/45	1/45	1/45	5/180 (2.8)
Copper (125 ppm)	0/45	19/45	8/45	11/45	38/180 (21.1)

tinomycin (MIC > 100 µg/ml) and *tet*(M) negative and sensitive to tetracycline (MIC = 0.39 µg/ml), were used as recipient strains. The donors and recipients were grown on BHI agar plates containing tetracycline (40 µg/ml) and spectinomycin (500 µg/ml), respectively. The resultant transconjugants were selected on BHI agar plates containing both tetracycline and spectinomycin. The transconjugants were tested for *tcrB* and *erm*(B) genes by PCR and their susceptibilities to copper were determined as described above. The transfer frequency for each strain was calculated as the CFU of transconjugants per recipient CFU.

Statistical analysis. The *tcrB* gene prevalence in treatment groups was analyzed using a generalized mixed model (PROC GLIMMIX, SAS, Version 9.2). The statistical model included the fixed effect of dietary treatment (control or elevated copper) with pen as a random effect. The analysis was performed to determine the effects both of sampling days and copper supplementation. Copper and vancomycin MIC values were analyzed for evidence of normal distribution (PROC UNIVARIATE) within and between *tcrB*-positive and -negative isolates. The MIC values were transformed based on rank (PROC RANK), and analysis of variance (PROC GLIMMIX) was performed on the ranked values since the MIC values were not normally distributed ($P < 0.05$).

RESULTS

Prevalence of the *tcrB*, *erm*(B), and *vanA* genes. A total of 360 enterococcal isolates consisting of 45 isolates (3 isolates per fecal sample, 3 piglets sampled per pen, and 5 pens per treatment) per treatment group (control or copper group) and sampling day (days 0, 14, 28, and 42) were obtained. All 360 enterococcal isolates were screened for the *tcrB* gene and 43 (11.9%) isolates were positive (amplicon size of 663 bp). Out of 43 *tcrB*-positive isolates, 5 (5/180; 2.8%) were from piglets fed the basal diet with a normal amount of copper (16.5 ppm) and 38 isolates (38/180; 21.1%) were from piglets fed the basal diet supplemented with an elevated amount of copper (Table 1). The overall prevalence of *tcrB*-positive enterococcal isolates was higher ($P < 0.05$) in the copper-supplemented group than in the control group. The prevalence of *tcrB* was affected by sampling day ($P < 0.05$) and also exhibited a significant treatment and sampling time interaction ($P < 0.05$). The 43 *tcrB*-positive and a subset of *tcrB*-negative isolates ($n = 44$) randomly chosen from the control group were used for the species identification. Based on multiplex PCR and *sodA* gene sequence analyses, 35 *tcrB*-positive isolates were *E. faecium* and 8 isolates were *E. faecalis*. Among 44 *tcrB*-negative isolates, 25 isolates were *E. faecium* and 19 isolates were *E. faecalis*. All *tcrB*-positive ($n = 43$) and *tcrB*-negative ($n = 44$) isolates were positive for the *erm*(B) gene and negative for the *vanA* gene.

MICs of copper, erythromycin, and vancomycin. All *tcrB*-positive isolates (35 *E. faecium* and 8 *E. faecalis*) grew on MH agar containing copper at 16 or 20 mM. However, the *tcrB*-negative isolates (25 *E. faecium* and 19 *E. faecalis* isolates) grew only at a 2 to 8 mM copper concentration. The mean MIC values of copper for *tcrB*-negative and *tcrB*-positive isolates were 6.2 and 22.2 mM, respectively, and the difference was significant at $P < 0.001$. The two reference strains obtained from Denmark (7430162-6 and 7430275-4) had MICs of 23 and 24 mM, respectively.

All *tcrB*-positive and *tcrB*-negative isolates were resistant to erythromycin (MIC > 100 µg/ml) and susceptible to vancomycin. The MIC values for vancomycin were higher ($P < 0.001$) for the *tcrB*-positive isolates (0.42 µg/ml) than for the *tcrB*-negative isolates (0.22 µg/ml); however, none of the isolates can be considered "resistant" using the breakpoint criterion (11).

PFGE typing. The clonal relationship among the 43 *tcrB*-positive isolates was determined (Fig. 1) by PFGE typing. Based on the species-specific distribution of PFGE patterns within and between the copper group and the control group, a total of 17 PFGE types were obtained (<95% Dice similarity) among the 35 *tcrB*-positive *E. faecium* isolates while the 8 *tcrB*-positive *E. faecalis* isolates showed 4 PFGE types. Among the five *tcrB*-positive isolates from the control group, three *E. faecalis* isolates belonged to two different PFGE types and the two *E. faecium* isolates were of different PFGE types. Among the 38 *tcrB*-positive isolates obtained from the copper group, 33 *E. faecium* isolates belonged to 15 different PFGE types and five *E. faecalis* isolates shared two PFGE patterns. Six *E. faecium* isolates (type 13) with identical banding patterns (100% Dice similarity) were obtained from four pens in the copper group on sampling day 14. Similarly, four *E. faecium* isolates (type 4) with identical banding patterns (100% Dice similarity) were obtained from two different pens in the copper group on sampling day 14. On day 28, three *E. faecalis* isolates obtained from the same pen had identical banding patterns. Among the two *tcrB*-positive *E. faecium* isolates in the control group, one isolate had a PFGE pattern identical to that of two *tcrB*-positive isolates (from the same pen) in the copper group. All *tcrB*-positive isolates obtained on three different sampling days (14, 28, and 42) belonged to different PFGE types except for two *E. faecium* isolates obtained from the same pen on day 28 matched with another isolate (from a different pen) obtained on day 42. Few of the *tcrB*-positive isolates obtained within each pen over three different sampling days (14, 28, and 42) belonged to different PFGE types. Among the isolates obtained from day 14, one PFGE type was distributed among three different pens, which was matched with other two different PFGE types shared between pens. On day 28, we observed only one PFGE that was common between two pens. In one pen (pen 32) that had 12 *tcrB*-positive isolates obtained on three different sampling days, there were six different PFGE types. In pens 7 and 13, which had seven *tcrB*-positive isolates each, there were five and six different PFGE types, respectively. Pen 21 with nine *tcrB*-positive isolates had five different PFGE types. Pen 10 with two *tcrB*-positive isolates had two PFGE types. In one pen of the copper group that had five *tcrB*-positive *E. faecium* isolates on sampling day 42, four had identical PFGE banding patterns.

Southern hybridization. The restriction digestion of the genomic DNA of *E. faecium* or *E. faecalis* with S1 nuclease yielded a distinct band of ~194-kbp size (Fig. 2). The restriction digestion of *tcrB*-negative *E. faecium* or *E. faecalis* with S1 nuclease did not yield 194-kbp bands but showed bands that ranged from 150 to 170 kbp in size (data not shown). In all
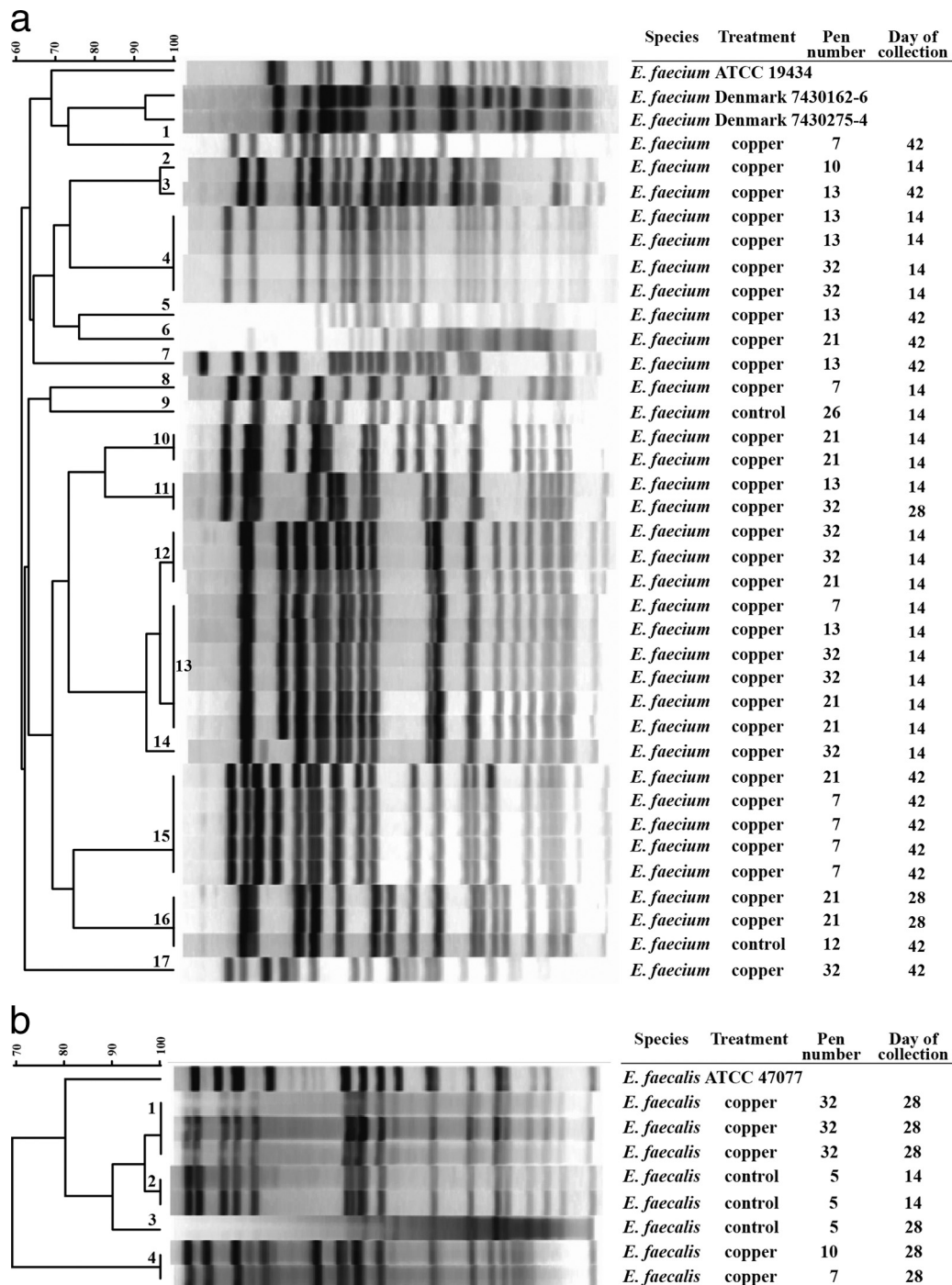
FIG. 1. (a) Pulsed-field gel electrophoresis banding patterns of SmaI-digested genomic DNA of *tcrB*-positive *Enterococcus faecium* isolates from piglets fed diets supplemented with or without copper. (b) Pulsed-field gel electrophoresis banding patterns of SmaI-digested genomic DNA of *tcrB*-positive *Enterococcus faecalis* isolates from piglets fed diets supplemented with or without copper.

three *tcrB*-positive *E. faecium* and *E. faecalis* isolates tested, both *tcrB* and the *erm*(B) gene probes hybridized with the ~194-kbp band (Fig. 2). In case of *tcrB*-negative *E. faecium* or *E. faecalis* strains, as expected, no hybridization was observed with *tcrB* gene probe; however, the *erm*(B) gene probe hybridized with 150- to 170-kbp bands (data not shown).

Interspecies transferability of *tcrB* gene. Five each of *tcrB*-positive *E. faecium* and *E. faecalis* isolates were used to demonstrate the interspecies transferability of *tcrB* gene by conjugation. The 10 resultant transconjugants were positive for *tcrB*, *erm*(B), and *tet*(M) genes. As expected, the transconjugants grew on MH agar containing high copper concentrations (16 or
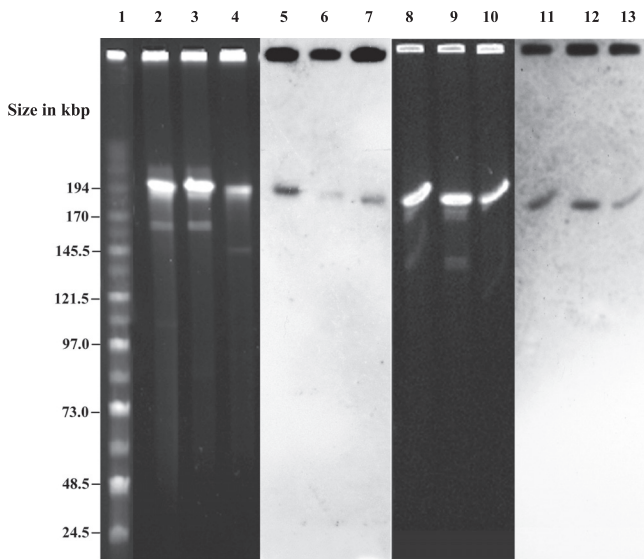
FIG. 2. Hybridization of *tcrB* and *erm*(B) probes to S1 nuclease-digested genomic DNA of three enterococcal strains. Lanes: 1, mid-range PFG marker II; 2 and 3, genomic DNA of *E. faecium* isolates digested with S1 nuclease enzyme; 4, genomic DNA of *E. faecalis* isolate digested with S1 nuclease enzyme; 5, 6, and 7, Southern blot hybridization with *tcrB* probe; 8 and 9, genomic DNA of *E. faecium* isolates digested with S1 nuclease enzyme; 10, genomic DNA of *E. faecalis* isolate digested with S1 nuclease enzyme; 11, 12, and 13, Southern blot hybridization with *erm*(B) probe.

20 mM). The mean copper MIC of the 10 transconjugants was 18.4 mM. The mean transfer frequencies for *tcrB*-positive *E. faecium* and *E. faecalis* isolates were 9.3×10^{-6} and 8.2×10^{-6}, respectively (Table 2).

DISCUSSION

In the present study, we examined the effect of feeding an elevated concentration of copper on the prevalence of *tcrB*-mediated, copper-resistant enterococcal isolates in the feces of piglets. Only a small proportion of fecal enterococcal isolates (11.9%; 43/360) obtained from piglets fed diets supplemented with normal or elevated levels of copper harbored the *tcrB* gene. This suggests that acquired resistance to copper may not be widespread in enterococci. In contrast to our study, Hasman and Aarestrup (19) have reported a prevalence of 76% (45/59) of copper-resistant enterococcal isolates from pigs obtained at the time of slaughter in Denmark. The higher prevalence of *tcrB* gene in the Danish piglets may be related to higher levels (175 to 250 ppm) or longer durations (birth to slaughter) of copper supplementation (21). In our study, the *tcrB* gene was detected in only two species, *E. faecium* and *E. faecalis*, and the majority of the resistant isolates (35/43; 81%) were *E. faecium*. Our results are in agreement with Danish studies, where the prevalence of *tcrB* gene was highest among *E. faecium* swine isolates; however, the *tcrB* was also detected in *E. gallinarum*, *E. casseliflavus*, and *E. mundtii* (19, 21). Similar results have been reported from a recent Australian study, in which the highest prevalence of *tcrB* was observed among *E. faecium* and *E. faecalis* isolates; however, *tcrB* was also detected in *E. gallinarum*, *E. casseliflavus*, *E. durans*, and *E. mundtii* (33). Even

among the randomly chosen *tcrB*-negative isolates (44 isolates) that were subjected to species identification, we did not detect species other than *E. faecium* and *E. faecalis*. Studies conducted in swine in the United States have generally reported *E. cecorum*, *E. durans*, *E. faecalis*, *E. faecium*, *E. hirae*, and *E. malodoratus* as the dominant species (14, 48). Differences in species distribution may be attributable to factors such as age, diet, use of in-feed antibiotics, isolation methods employed, and geographical location (24). Because samples in our study were collected only during the nursery stage, the predominant species may be representative of the population at that stage of production (24).

In piglets, copper is added to diets at concentrations above those physiologically required by the animal because of its growth-promoting effects (38). The exact mechanism of action of copper as a growth promoter has not been elucidated, but suggested mechanisms attributed to the antimicrobial effects of copper include altered gut microbial populations, increased availability of nutrients and energy because of the reduced microbial activity in the gut, and possibly the suppression of enteric bacterial pathogens (18, 23). In European countries, copper is supplemented in swine diets at elevated levels, often as a replacement for in-feed antibiotics, which have been banned for use as growth promotants since the mid-1990s (3, 21).

The higher prevalence of *tcrB* in enterococci from piglets fed an elevated level of copper compared to the control group (21.1 versus 2.8%) suggests the exertion of selection pressure for copper resistance. The presence of *tcrB*-positive isolates in the control group fed normal level of copper in the diet suggests the occurrence of naturally resistant isolates not favored by a high level of copper in the diet. Only one isolate (among five) of *E. faecium* in the control group was clonally identical to an isolate from the copper group, suggesting that the occurrence was not because of the transmission of *tcrB*-positive strains from the copper-treated group. We obtained three enterococcal isolates per sample (nine per pen), which allowed us to screen 45 isolates per treatment group and sampling day. Although the presence of *tcrB*-positive isolates was detected only after the initiation of copper supplementation, we did not find a linear increase in the prevalence with continued supplementation of an elevated level of copper. In fact, the highest prevalence was on day 14 of copper supplementation. The PFGE analysis of *tcrB*-positive isolates revealed that there was no dominant or persistent PFGE type in the copper-supple-

TABLE 2. Transfer frequency of *tcrB* gene in *Enterococcus faecium* and *Enterococcus faecalis*

Donor strain	Recipient strain	Transfer frequency (no. of transconjugants per recipient)
E. faecium KSU 143	*E. faecalis* OG1SSp	1.18×10^{-5}
E. faecium KSU 150	*E. faecalis* OG1SSp	7.88×10^{-6}
E. faecium KSU 153	*E. faecalis* OG1SSp	6.97×10^{-6}
E. faecium KSU 160	*E. faecalis* OG1SSp	9.48×10^{-6}
E. faecium KSU 167	*E. faecalis* OG1SSp	1.02×10^{-5}
E. faecalis KSU 101	*E. faecium* TX5034	1.05×10^{-5}
E. faecalis KSU 102	*E. faecium* TX5034	8.15×10^{-6}
E. faecalis KSU 201	*E. faecium* TX5034	7.43×10^{-6}
E. faecalis KSU 234	*E. faecium* TX5034	7.67×10^{-6}
E. faecalis KSU 269	*E. faecium* TX5034	6.96×10^{-6}

mented group. Almost all isolates obtained on different sampling days (14 days apart) belonged to different PFGE types. Based on an *in vivo* pig challenge study, Hasman et al. (21) reported that feeding elevated level of copper resulted in the selection of *tcrB*-mediated copper-resistant *E. faecium*. The *in vivo* challenge study was done with piglets fed low-copper (6 ppm) or high-copper diets (175 ppm), which were orally inoculated with *tcrB*-positive (copper-resistant) and *tcrB*-negative (copper-sensitive) *E. faecium* strains. The fecal prevalence of *tcrB*-positive *E. faecium* was significantly higher in the copper-supplemented group (175 ppm copper) than in the control (6 ppm copper).

Hasman and Aarestrup (19) have reported on the isolation of a large plasmid (~175 kb) from a copper-resistant *E. faecium* isolate from a pig in Denmark. The plasmid was transferable and carried genes for copper, macrolide, and glycopeptide resistance. Because the isolation of a large plasmid with a low copy number from a Gram-positive bacterium is difficult, we used S1 nuclease to facilitate the detection of the large plasmid DNA (8). The S1 nuclease attacks only a single-stranded DNA (50, 41), leading to the conversion of supercoiled plasmid DNA into a full-length linear molecule (8). The S1 nuclease digestion of genomic DNA of three randomly chosen *tcrB*-positive strains of *E. faecium* or *E. faecalis* yielded bands of approximately 194-kb size to which both *tcrB* and *erm*(B) gene probes hybridized, suggesting their colocation on the same plasmid. The *tcrB*-negative strains of *E. faecium* or *E. faecalis* that we tested did not carry the 194-kbp plasmid but had plasmids that ranged from 150 to 170 kbp in size, which carried the *erm*(B) gene. The presence of the *erm*(B) gene on plasmids of different sizes have been reported (22, 25). In enterococci of human origin, *vanA*-type glycopeptide resistance and other antibiotic resistance determinants have been shown to be located on a plasmid of about 100 kb in size (49). The large plasmids of enterococci may play a major role in the horizontal transfer of resistance determinants in enterococci (30).

An interesting aspect of copper resistance in enterococci of piglets is the possible linkage to macrolide and glycopeptide resistance because of colocalization of *tcrB* and *erm*(B) genes on the same plasmid (19). Hasman et al. (21) have shown that elevated levels of copper fed to piglets coselected for erythromycin and vancomycin resistance in enterococcal isolates obtained in Denmark and Spain. More often, the antibiotic and heavy metal resistance genes are located on the same mobile genetic elements, such as plasmids, transposons, and integrons (5, 39). Therefore, there is a possibility that the natural selection pressure imposed by heavy metals in the feed may indirectly coselect for antibiotic resistance. We were unable to assess the coselection because in our study, both *tcrB*-positive and *tcrB*-negative isolates were phenotypically resistant to erythromycin (MIC > 100 μg/ml) and contained the *erm*(B) gene. The *erm*(B) gene is the most common determinant associated with enterococcal resistance to macrolides (25) and tylosin, an erythromycin derivative, is used in the feed of piglets in the United States to treat enteric infections and also for growth promotion (25). Not surprisingly, our *tcrB*-positive isolates did not carry the *vanA* gene and were susceptible to vancomycin (MIC = 0.42 μg/ml). However, the vancomycin MIC values were higher for *tcrB*-positive isolates than for *tcrB*-

negative isolates. The reason for the difference is not known: possibly a nonspecific shared resistance mechanism(s) imparted by the *tcrB* gene is likely responsible for the difference in susceptibility to vancomycin (7). However, the biological implication of the difference in susceptibility to vancomycin is not known. The prevalence of *vanA* in enterococcal isolates of piglets and chickens in Europe was likely because of the use of avoparcin, a glycopeptide, for growth promotion (6). The use of avoparcin was banned in Denmark in 1995 and in the European Union in 1997 (1, 3). The absence of glycopeptide resistance in swine enterococcal isolates in our study is likely because avoparcin or other related glycopeptide derivatives have never been used in the swine industry in the United States (12, 34). However, isolation of vancomycin-resistant *E. faecium* (VRE) containing *vanA* has recently been reported from swine in Michigan (15). The source of VRE acquisition was not determined in the study, but there was no association between the occurrence of VRE and antibiotic administration in the pigs.

Antibiotic-resistant enterococci are opportunistic and nosocomial pathogens in humans (35). Because of the widespread occurrence of resistant enterococci in animals, it has been suggested that these enterococci may serve as a reservoir of potential resistant genes capable of transferring from animal to human bacteria (28). The importance of enterococci is related to their propensity for conjugal transfer of resistance genes, mostly associated with the plasmids or transposons. In our previous study (4), we demonstrated the transferability of the *tcrB* gene between strains within the same species of enterococci. In the present study, we have demonstrated the interspecies transferability of the *tcrB* gene between *E. faecium* and *E. faecalis*. The efficient transfer of genes by conjugation indicates the presence of conjugative plasmids and or other mobile genetic elements (13, 40). The transconjugants possessing *erm*(B) and *tcrB* genes were indicative of their presence on a conjugative plasmid. The resistance determinants *tet*(M) and *erm*(B) are frequently carried on conjugative transposons Tn*916* and Tn*916*/Tn*1545* (10). These conjugative transposons have a broad bacterial host range and are capable of being transferred horizontally to a variety of Gram-positive and Gram-negative bacteria in the gut bacterial community (9). A critical issue is the coselection of metal and antibiotic resistance on the same genetic determinant, often on a plasmid or a transposon (37). These plasmids or transposons are of concern because they harbor antibiotic resistance genes and have the potential to spread between species (37). The existence of a metal-associated coselection mechanism could potentially be a major issue relative to public health, and further studies are needed to determine the magnitude of such an association. In conclusion, our longitudinal study showed a relationship between elevated levels of copper supplementation and the prevalence of copper-resistant enterococci in piglets. Also, we have confirmed the presence of the *tcrB* and *erm*(B) genes on a plasmid, suggesting potential transferability and coselection.

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