APPLIED AND ENVIRONMENTAL MICROBIOLOGY, May 2008, p. 2950–2956 Vol. 74, No. 10
0099-2240/08/$08.00+0 doi:10.1128/AEM.00189-08

Collateral Effects of Antibiotics: Carbadox and Metronidazole Induce VSH-1 and Facilitate Gene Transfer among *Brachyspira hyodysenteriae* Strains▽

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Received 21 January 2008/Accepted 6 March 2008

Brachyspira hyodysenteriae **is an anaerobic spirochete and the etiologic agent of swine dysentery. The genome of this spirochete contains a mitomycin C-inducible, prophage-like gene transfer agent designated VSH-1. VSH-1 particles package random 7.5-kb fragments of the *B. hyodysenteriae* genome and transfer genes between *B. hyodysenteriae* cells. The chemicals and conditions inducing VSH-1 production are largely unknown. Antibiotics used in swine management and stressors inducing traditional prophages might induce VSH-1 and thereby stimulate lateral gene transfer between *B. hyodysenteriae* cells. In these studies, VSH-1 induction was initially detected by a quantitative real-time reverse transcriptase PCR assay evaluating increased transcription of *hvp38* (VSH-1 head protein gene). VSH-1 induction was confirmed by detecting VSH-1-associated 7.5-kb DNA and VSH-1 particles in *B. hyodysenteriae* cultures. Nine antibiotics (chlortetracycline, lincomycin, tylosin, tiamulin, virginiamycin, ampicillin, ceftriaxone, vancomycin, and florfenicol) at concentrations affecting *B. hyodysenteriae* growth did not induce VSH-1 production. By contrast, VSH-1 was detected in *B. hyodysenteriae* cultures treated with mitomycin C (10 μg/ml), carbadox (0.5 μg/ml), metronidazole (0.5 μg/ml), and H_2O_2 (300 μM). Carbadox- and metronidazole-induced VSH-1 particles transmitted tylosin and chloramphenicol resistance determinants between *B. hyodysenteriae* strains. The results of these studies suggest that certain antibiotics may induce the production of prophage or prophage-like elements by intestinal bacteria and thereby impact intestinal microbial ecology.**

In the United States, various antimicrobials are added to feed to prevent diseases and to promote growth or to enhance the feeding efficiency of swine (14, 28). Antibiotics commonly used in feed for swine include tetracyclines, carbadox, macrolides, and lincosamides (19). At higher concentrations, carbadox, lincomycin, tylosin, and tiamulin are added to feed or drinking water for the treatment of swine intestinal diseases, notably swine dysentery (25, 28). In Australia and some European countries, nitroimidazole antibiotics, such as metronidazole, ronidazole, and dimetridazole, have been used to treat swine dysentery (22, 25; D. Trott, personal communication), although legislation in several countries has restricted the use of these antibiotics in food animals (2, 44).

The etiologic agent of swine dysentery is the anaerobic spirochete *Brachyspira hyodysenteriae*. Within their genome, *B. hyodysenteriae* cells carry a mitomycin C-inducible prophage-like element, designated VSH-1 (30, 31, 63). Unlike traditional prophages, VSH-1 particles contain random 7.5-kb fragments of the *B. hyodysenteriae* genome. VSH-1 head, tail, and lysis genes total at least 16.3 kb of DNA (38). Consequently, an individual VSH-1 particle is incapable of lytic growth, and there are no bioassays (i.e., plaque formation) for measuring VSH-1 production. Although VSH-1 particles do not self-propagate, they transfer genes between *B. hyodysenteriae* cells (31, 54). These unusual properties of VSH-1 are shared by similar elements in species of *Rhodobacter*, *Methanococcus*, and *Desulfovibrio* (37, 52). VSH-1 and the other elements have collectively been designated gene transfer agents (GTAs) (15).

One goal of our research is to identify environmental inducers of VSH-1 production. *B. hyodysenteriae* cells are undoubtedly exposed to antimicrobials in the swine intestinal tract or in the farm environment. In these investigations, different antibiotics were tested as inducers of VSH-1. Carbadox and metronidazole were potent VSH-1 inducers. VSH-1 virions induced by these antibiotics transferred chloramphenicol resistance and tylosin resistance between *B. hyodysenteriae* cells.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The *B. hyodysenteriae* strains $B78^T$ (type strain), B204, and A203 were used in these studies. Strain $B78^T$ is sensitive (MIC = 4 to 8 μg/ml) to the macrolide antibiotic tylosin (33). Strain B204 is naturally resistant to tylosin (Ty^r) (MIC > 256 μg/ml) due to a 23S rRNA mutation, T in place of A_{2058} (33). Strain A203 is chloramphenicol resistant (Cm^r) (MIC > 10 μg/ml) and was derived from strain B204 by inserting a *cat* cassette into the *flaA1* gene (31, 46).

B. hyodysenteriae cells were routinely grown at 38°C in stirred 10-ml BHIS broth cultures beneath an initial N_2-O_2 (99:1) culture atmosphere (53). BHIS broth is anaerobically prepared brain heart infusion broth supplemented with 10% (vol/vol) heat-treated calf serum. (For unknown reasons, the BD Bacto brand of BHI was superior to BHI obtained from BBL for optimum induction of VSH-1.) All cultures were in the exponential phase of growth at the time of use (optical density at 620 nm [OD_{620}] = 0.5 to 1.0; 18-mm path length). VSH-1

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▽ Published ahead of print on 21 March 2008.

particles for antibiotic resistance transfer experiments were obtained from *B. hyodysenteriae* A203 cells cultured in NT broth, a serum-free, low-protein-content medium essential for VSH-1 purification (31). Culture media for strain A203 cells contained chloramphenicol (10-μg/ml final concentration).

Identification of VSH-1 inducers and their effects on cell growth. *B. hyodysenteriae* B204 cultures in early exponential growth phase (OD_{620} = 0.5) in BHIS broth were treated with potential VSH-1 inducers. The culture ODs were monitored over time to detect differences in cell growth between treated and untreated (control) cultures. Specific treatments impairing or inhibiting growth (defined below) were subsequently tested for the ability to stimulate VSH-1 *hvp38* transcription.

Antibiotics tested for their growth effects were as follows (range of final concentrations in μg/ml of BHIS culture): carbadox (0.005 to 0.2), metronidazole (0.005 to 2), chlortetracycline (5 to 50), lincomycin (10 to 100), tylosin (500 to 1,000), tiamulin (0.02 to 0.2), virginiamycin (0.1 to 15), ampicillin (0.5 to 30), ceftriaxone (5 to 100), vancomycin (500 to 1,000), and florfenicol (2 to 20). Within the concentration ranges, two- or fourfold dilutions of antibiotics were tested. Stock solutions or suspensions (50 to 100×) were prepared in sterile water, because ethanol was a weak inducer of VSH-1. In each experiment, parallel control cultures were either untreated or treated with 10 μg mitomycin C/ml (30).

RNA purification. Six hours after treatment with potential inducers of VSH-1, samples (2 to 5 ml) of *B. hyodysenteriae* cultures were diluted 1:3 in RNA Protect (Qiagen, Valencia, CA). After 5 min at room temperature, spirochete cells were harvested by centrifugation (5,000 × *g*; 5 min), and the cell pellets were stored frozen (−85°C) for up to 2 weeks. Total RNA was extracted from the cell pellets by using RNAeasy minicolumn kits following the manufacturer's instructions (Qiagen). DNA was removed by treating the RNA preparations with Turbo DNase following the manufacturer's instructions (Applied Biosystems/Ambion, Foster City, CA). RNA concentrations were estimated spectrophotometrically (OD_{260}) by using microcapillary cuvettes and a Beckmann DU-650 spectrophotometer and were based on standard conversion values (49). This protocol yielded 1 to 2 μg of RNA/ml of culture.

The purity and quality of RNA preparations were assessed by examining the banding patterns after gel electrophoresis of 300 ng of RNA (4% Nu-Sieve agarose; 1× Tris-borate-EDTA). RNA solutions were diluted in nuclease-free water (Integrated DNA Technologies, Coralville, IA) to a final concentration of 5 ng/ml, dispensed in aliquots, and frozen (−85°C) until they were used.

Identification of VSH-1 inducers by QRT-PCR of *hvp38* transcription. Quantitative real-time reverse transcriptase PCR (QRT-PCR) was used to detect increases in mRNA transcribed from the VSH-1 *hvp38* gene. The *hvp38* gene encodes the VSH-1 major capsid protein, and increased *hvp38* transcription is an early event in VSH-1 induction by mitomycin C (38, 39). QRT-PCR was also used to determine the 16S rRNA content of each RNA preparation, and this value was used as an internal reference standard against which relative changes in *hvp38* mRNAs were calculated.

Based on the *hvp38* sequence (GenBank accession no. AY971355), the primers for amplifying *hvp38* mRNA were 38F (5′-TTCAAGACTTGGGCTTTTAAGAG) and 38R (5′-TTGGTTTGGCACTTAAATCAAC), and the probe was 5′-GTGTTTGTGCTTCCATAAAGTTCTGCATCTGT. Based on the *B. hyodysenteriae rrs* sequence (GenBank accession no. M57741), the primers for amplifying 16S rRNA were 16F (5′-TCATGGCCCTTATGTCCAG) and 16R (5′-CGAACTGAGGCAACTTTTTTG), and the probe was 5′-CACGTGCTACAATGGCAAGTACAAAGAGA. Probes were labeled at the 3′ end with the quencher dye TAMRA (6-carboxytetramethylrhodamine). The *hvp38* probe was labeled at the 5′ end with VIC (PE Biosystems, Foster City, CA), and the 16S rRNA probe was labeled with TET (6-carboxy-2′,4,7,7′-tetracholorofluorescein). Primers and probes were designed using Primer Express software (PE Applied Biosystems, Foster City, CA) and were synthesized by Integrated DNA Technologies.

For QRT-PCR analysis, 1 pg of total RNA was used for 16S rRNA amplification and 1 ng for *hvp38* mRNA amplification. A reaction mixture contained 2 μl of target RNA, 15 pmol of each primer, and 0.126 pmol of probe added to a TaqMan One Step Master Mix reagent kit (Applied Bioystems) in a final volume of 25 μl.

Amplification and detection were carried out in optical-grade 96-well plates in an ABI Prism 7700 sequence detection system (PE Biosystems). Following an initial RT-PCR consisting of 30 min of incubation at 48°C for converting mRNA to cDNA, amplification of cDNA was carried out with one cycle of 95°C for 10 min, followed by 40 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 60 s. The final extension was carried out at 72°C for 2 min, followed by cooling of samples to 25°C.

Copies of the *B. hyodysenteriae rrs* (16S rRNA) and *hvp38* genes cloned in pBlueScript II SK(−) (Stratagene, La Jolla, CA) were used as templates to generate RNA gene transcripts using the RNAMaxx high-yield transcription kit (Stratagene). Standard curves were generated for estimating 16S rRNA and *hvp38* mRNA levels in cells by linear regression analysis.

In each experiment, RNA levels were assayed in triplicate for at least two cultures. Control amplifications containing known quantities (copy numbers) of *rrs* and *hvp38* RNAs were included. Control amplifications lacking reverse transcriptase were included for each RNA preparation to confirm that *B. hyodysenteriae* DNA was absent from the sample.

Identification of VSH-1 inducers and detection of VSH-1 7.5-kb DNA. *B. hyodysenteriae* cells in cultures (5 ml) exposed to potential VSH-1 inducers were harvested by centrifugation and resuspended in 0.35 ml of 50 mM Tris, pH 8.0, containing 0.05 mM EDTA. Lysozyme (0.1 ml of a 10-mg/ml solution in 0.25 mM Tris, pH 8) was added, and the cell suspension was placed on ice for 45 min. Proteinase K (0.1 ml of a solution containing 0.1 mg proteinase K, 0.5% sodium dodecyl sulfate, 50 mM Tris, pH 7.5, 10 mM EDTA, 50 μM $CaCl_2$) was then added.

After incubation at 50°C for 1 h, the solution was extracted twice, first with an equal volume of Tris-buffered phenol-chloroform–isoamyl alcohol (Sigma-Aldrich, St. Louis, MO) and then with $CHCl_3$. Phase Lock 1 tubes (Eppendorf) were used to separate the extraction phases. DNA in the final aqueous phase was precipitated with 3 M sodium acetate-ethanol (49), air dried, and dissolved in 0.5 ml of 50 mM Tris, pH 7.4, containing 1 mM EDTA. After 1 h of RNase (RNAce it; Stratagene) digestion, the DNA was again solvent extracted, precipitated with acetate-ethanol, and dissolved in 50 μl of Tris-EDTA buffer, as described above. This method yielded 5 to 25 μg of DNA, as determined spectrophotometrically using microcapillary cuvettes (Beckman DU650 spectrophotometer).

Identification of VSH-1 inducers by electron microscopy. *B. hyodysenteriae* cells exposed to potential inducers of VSH-1 were examined by electron microscopy to detect VSH-1 particles as described previously (30). Up to 50 microscope fields containing well-stained spirochete cells were examined. Negative control cultures (untreated) and positive control cultures (treated with mitomycin C or carbadox) were examined in parallel.

VSH-1 transfer of tylosin and chloramphenicol resistances. NT broth cultures of *B. hyodysenteriae* strain A203 (Cm^r Ty^r; OD_{620} = 0.5) were treated with mitomycin C (20 μg/ml), metronidazole (1 μg/ml), or carbadox (1 μg/ml) to obtain VSH-1 particles. Five hours after induction, VSH-1 virions were harvested from 400-ml cultures and resuspended in SM buffer (31). Purified virions (10 μl containing 1 to 2.5 μg of DNA) were added to 10-ml BHIS broth cultures of *B. hyodysenteriae* B204 (Cm^s Ty^r) and strain $B78^T$ (Cm^s Ty^s) in exponential growth phase (~5×10^7 cells/ml). After overnight incubation at 39°C, the cultures (OD_{620} = 1.0; 3×10^8 to 5×10^8 CFU/ml) were transferred into a Coy anaerobic chamber inflated with a gas mixture of 85% N_2-10% CO_2-5% H_2. The cultures were serially diluted 10-fold, and 0.1 ml of the dilutions was plated onto Trypticase soy blood (TSB), TSB plus tylosin (10-μg/ml final concentration), and TSB plus chloramphenicol (10 μg/ml) agar. TSB agar plates (54) had been placed into the Coy chamber 18 to 24 h before they were used. After 4 days of incubation at 39°C in the chamber, *B. hyodysenteriae* antibiotic-resistant colonies (hemolytic zones) were counted. Cm^r genotypes were confirmed by PCR detection of the *cat* gene (54). Ty^r genotypes were confirmed by sequence analysis of a PCR-amplified region of the 23S rRNA gene containing the $A_{2058} \rightarrow T$ mutation associated with resistance to macrolides (33). Control *B. hyodysenteriae* cultures for assessing spontaneously resistant mutants did not receive VSH-1.

RESULTS

Antibiotic concentrations affecting *B. hyodysenteriae* growth. *B. hyodysenteriae* B204 growth was inhibited (no increase in the culture OD) by 2 μg carbadox/ml (Fig. 1A). At 0.5 μg carbadox/ml, bacterial growth was first impaired (a slow increase in the OD), and then the culture OD declined between 3 and 5 h after exposure to the antibiotic. This decline in the OD_{620} in cultures treated with carbadox resembled that of a mitomycin C-treated culture during VSH-1 induction (30). Concentrations of 0.2 μg carbadox/ml or lower were subinhibitory for *B. hyodysenteriae* growth in BHIS broth (Fig. 1A).

In similar growth studies, different concentrations of antibiotics and 300 μM H_2O_2 were found to impair or inhibit *B. hyodysenteriae* growth in BHIS broth (Fig. 1B). Among these,

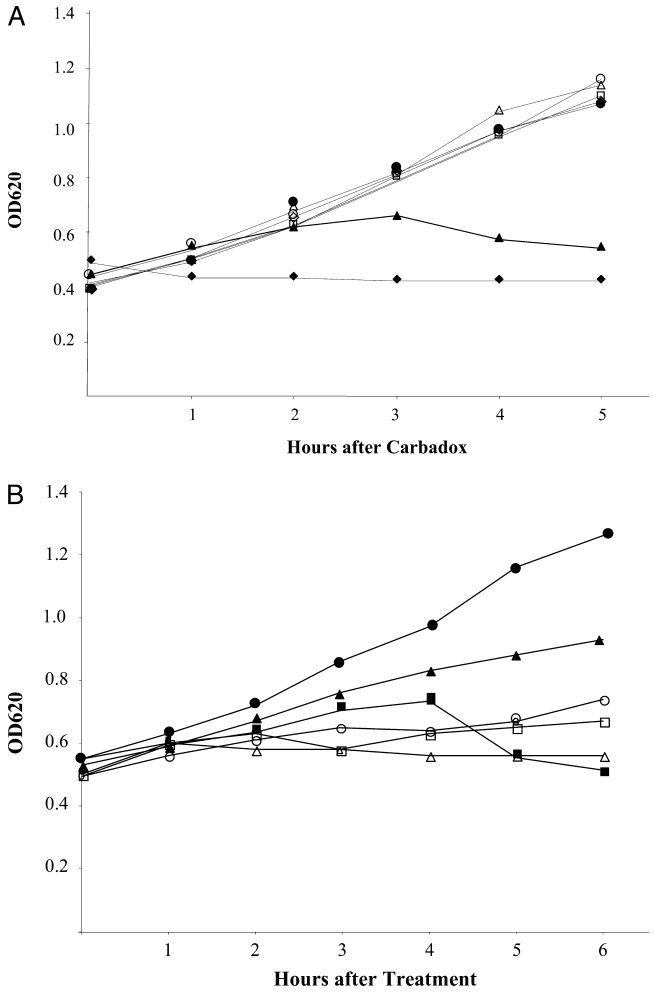


FIG. 1. (A) Carbadox effects on *B. hyodysenteriae* B204 growth. Carbadox was added (0 h) to cultures at final concentrations (μg/ml) of 0 (open circles), 0.005 (open triangles), 0.02 (open diamonds), 0.05 (open squares), 0.2 (closed circles), 0.5 (closed triangles), and 2.0 (closed diamonds). (B) Effects on *B. hyodysenteriae* B204 growth of no addition (control; closed circles), mitomycin C (10 μg/ml; closed squares), metronidazole (0.5 μg/ml; closed triangles), virginiamycin (0.5 μg/ml; open circles), tiamulin (0.1 μg/ml; open squares), and H_2O_2 (300-μM final concentration; open triangles).

only mitomycin C consistently produced a decline in the culture OD_{620}. *B. hyodysenteriae* cultures exposed to these and other concentrations were examined for induction of *hvp38* transcription.

Antibiotic induction of the GTA VSH-1. Chlortetracycline, lincomycin, tylosin, tiamulin, virginiamycin, ampicillin, ceftriaxone, vancomycin, and florfenicol, at the tested concentrations, did not stimulate *hvp38* transcription (Table 1) and thus were not inducers of VSH-1. Chlortetracycline, lincomycin, tylosin, tiamulin, and virginiamycin are approved medicated-feed additives for treating swine dysentery, for promoting growth/feeding efficiency, or for both applications (28). Florfenicol and ampicillin are approved for the treatment of swine diseases (14).

Transcription of *hvp38* in *B. hyodysenteriae* cultures treated with carbadox (0.5 μg/ml) or metronidazole (0.5 μg/ml) increased 290- and 720-fold, respectively, over that in untreated cultures (Table 1). VSH-1 production in treated cultures was confirmed by detecting both VSH-1-associated 7.5-kb DNA fragments (Table 1 and Fig. 2) and VSH-1 particles (Table 1 and Fig. 3). Carbadox at 2 μg/ml inhibited growth (Fig. 1A), and intact DNA could not be obtained from the cultures, presumably an indication of DNA degradation (Fig. 2).

Transcription of *hvp38* was induced by carbadox or metronidazole at concentrations ranging between 0.005 and 0.5 μg/ml (Table 1). VSH-1-associated DNA was detected in *B. hyodysenteriae* cultures treated with either antibiotic at concentrations between 0.05 and 0.5 μg/ml (Fig. 1 and 2), and particles were detected in cultures treated with 0.05 μg carbadox/ml. In cultures treated with 0.005 μg/ml of either carbadox or metronidazole, VSH-1 production was not directly detected, and if it occurred, was likely below the detection limits of the two assays.

Mitomycin C is commonly used to induce prophages and was used in experiments leading to the discovery of VSH-1 (30). Hydrogen peroxide can also induce bacteriophage production (20, 56, 58) and induces VSH-1 in vitro and possibly in the swine intestinal tract (39). Transcription of *hvp38* in *B. hyodysenteriae* cells treated with mitomycin C (10 μg/ml) or H_2O_2 (300 μM) increased 260-fold and 100-fold, respectively, over that in untreated cultures (Table 1). VSH-1-associated 7.5-kb DNA and particles were detected in H_2O_2-treated cultures (Table 1 and Fig. 2B), although VSH-1 particles were produced at much lower levels in these cultures than in carbadox-treated cultures. A previous study used semiquantitative RT-PCR and Northern slot blot techniques and demonstrated that H_2O_2 and mitomycin C induce transcription of *hvp38* and VSH-1 production (39). The present results support the findings of that study. The QRT-PCR method used in this study, however, is a more sensitive assay of *hvp38* transcription than the Northern slot blot technique and provides quantitative measurements useful for comparing inducing treatments.

TABLE 1. Antimicrobial compounds and conditions inducing VSH-1

Treatment	Antimicrobial concn		*hvp38* transcription (fold increase)[a]	VSH-1 production[b]	
	μg/ml	μM		DNA	EM
Control (untreated)			1	−	−
Mitomycin C	10	30	260	+	+
Metronidazole	0.5	2.5	720	+	+
	0.05	0.25	50	+	−
	0.005	0.025	7	−	−
Carbadox	0.5	1.9	290	+	+
	0.05	0.19	30	+	+
	0.005	0.019	14	−	−
H_2O_2		300	100	+	+

[a] Increase in *hvp38* transcription over untreated (control) cultures. Levels of *hvp38* mRNA in control cultures were arbitrarily standardized to a value of 1. An increase of 5-fold or greater in each of triplicate cultures undergoing a particular treatment was considered significant. The values are mean values for at least two experiments. The following antimicrobials at the indicated concentrations (μg/ml) were not found to stimulate *hvp38* transcription: chlortetracycline (5, 10, 50), lincomycin (10, 50, 100), tylosin (500, 1,000), tiamulin (0.02, 0.1), virginiamycin (3), ampicillin (3, 15), ceftriaxone (30), vancomycin (500, 1,000), and florfenicol (2, 10, 20).

[b] A culture was considered positive (+) for VSH-1 production if DNA extracted from spirochete cells contained a 7.5-kb band (Fig. 2) or if VSH-1 particles were detected by electron microscopy (EM) (Fig. 3).

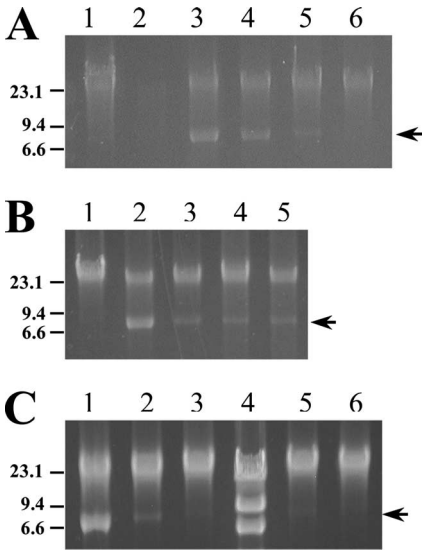


FIG. 2. Induction of VSH-1 7.5-kb DNA in *B. hyodysenteriae* B204 cultures. (A) DNAs from carbadox-treated cultures. Lane 1, control (untreated) culture; lane 2, carbadox (2 μg/ml)-treated culture; lane 3, carbadox (0.5 μg/ml); lane 4, carbadox (0.1 μg/ml); lane 5, carbadox (0.05 μg/ml); lane 6, carbadox (0.01 μg/ml). The absence of DNA in lane 2 is likely due to DNA degradation in spirochete cells. (B) DNAs from cultures treated with various antimicrobials. Lane 1, control (untreated) culture; lane 2, carbadox (0.5 μg/ml); lane 3, carbadox (0.1 μg/ml); lane 4, H_2O_2 (200 μM); lane 5, H_2O_2 (300 μM). (C) DNAs from *B. hyodysenteriae* cultures treated with carbadox or metronidazole. Lane 1, carbadox (0.5 μg/ml); lane 2, carbadox (0.05 μg/ml); lane 3, carbadox (0.005 μg/ml); lane 4, HindIII-digested lambda size markers; lane 5, metronidazole (0.05 μg/ml); lane 6, metronidazole (0.005 μg/ml). DNA sizes estimated from markers (kb) are indicated to the left of the panels. The arrows on the right of the panels indicate the positions of VSH-1-associated DNA (7.5 kb). Each lane contained 0.5 μg of DNA extracted from a *B. hyodysenteriae* culture.

While electron microscopy is the most reliable method for detecting VSH-1 production, it is, unfortunately, the least sensitive and most labor-intensive. Attempts to develop a VSH-1 bioassay (by measuring gene transfer frequencies) in our laboratory have been unsuccessful.

Gene transfer by metronidazole- or carbadox-induced VSH-1. To determine whether antimicrobial-induced VSH-1 particles were capable of transferring genes between *B. hyodysenteriae* strains, VSH-1 particles were purified from *B. hyodysenteriae* A203 (Ty^r Cm^r) cultures treated with carbadox or metronidazole. The VSH-1 particles were added to cultures of *B. hyodysenteriae* $B78^T$ (Ty^s Cm^s). After overnight incubation, strains resistant to either tylosin or chloramphenicol were present in cultures that received VSH-1 particles and were not detected or were beneath the limit of detection in control cultures (Table 2). Transductant cells doubly resistant to both antibiotics were not recovered.

Genotype analysis of six randomly selected chloramphenicol-resistant transductants from two experiments were examined, and all contained the *cat* gene of strain A203 (Fig. 4A). Eight randomly selected tylosin-resistant transductants had the same A_{2058}→T 23S rRNA gene base modification as the *B. hyodysenteriae* A203 strain from which VSH-1 had been induced (Fig. 4B).

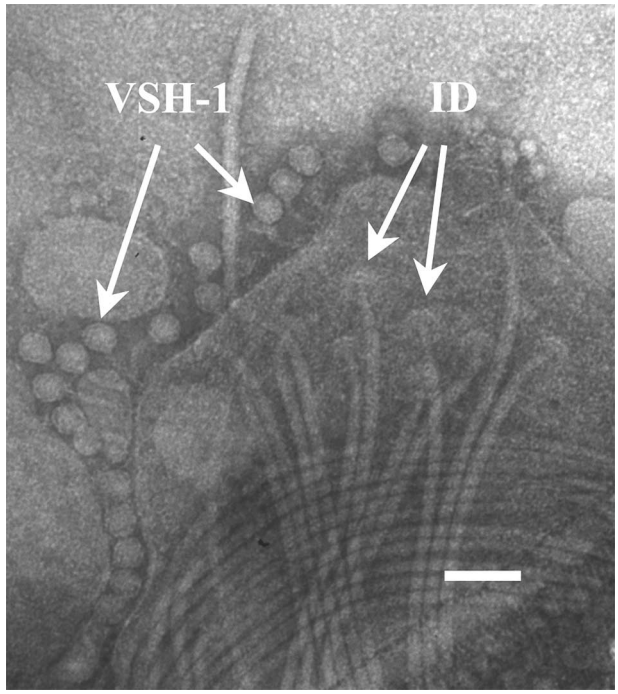


FIG. 3. Transmission electron micrograph of VSH-1 particles (VSH-1) surrounding a disrupted *B. hyodysenteriae* B204 cell from a culture treated with 0.5 μg carbadox/ml. Flagella and flagellum insertion disks (ID) are visible. Phosphotungstic acid stain (2%; pH 6.5). Marker bar = 0.1 μm.

DISCUSSION

Antimicrobials can have effects on target bacteria, in addition to their bactericidal or bacteriostatic effects. Different antibiotics have been found to modulate gene transcription in *Salmonella enterica* (23) and *Escherichia coli* (27), to induce *Pseudomonas aeruginosa* biofilm formation (29), and to stimulate toxin production by *Staphylococcus aureus* (42) and *E. coli* (24, 59, 61). These "collateral" effects are manifested at antibiotic concentrations subinhibitory for bacterial growth.

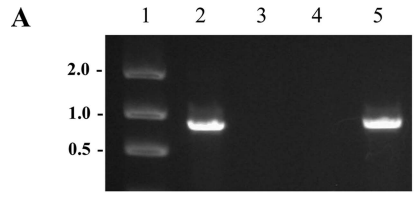
Although from a clinical perspective bacterial exposure to

TABLE 2. VSH-1-mediated transduction of chloramphenicol and tylosin resistances between *B. hyodysenteriae* strains A203 and B78

VSH-1 virion	No. of transductants/10^8 CFU[a]	
	Tylosin resistant (10 μg/ml)	Chloramphenicol resistant (10 μg/ml)
None (control)	<LOD[b]	<LOD
Mitomycin C induced	470	800
Metronidazole induced	405	390
Carbadox induced	250	820

[a] VSH-1 virions were induced by treating NT cultures of *B. hyodysenteriae* strain A203 (Ty^r Cm^r) with mitomycin C (20 μg/ml), metronidazole (1 μg/ml), or carbadox (1 μg/ml). Purified virions (containing 1 to 2.5 μg DNA) were added to *B. hyodysenteriae* B78 (Ty^s Cm^s) cultures. The values represent average numbers of resistant CFU per total numbers of CFU as determined in two experiments using two VSH-1 preparations for each treatment.

[b] LOD, limit of detection. Spontaneously occurring tylosin-resistant cells were too few for accurate estimates (i.e., 2 to 4 CFU/10^8 CFU). Spontaneously occurring chloramphenicol-resistant cells were not detected.



B Seq #1 ...AACAGGACGGT$_{2058}$AAGACCCCGTG...

Seq #2 ...AACAGGACGGA$_{2058}$AAGACCCCGTG...

FIG. 4. Genotype analyses of antibiotic resistance determinants of *B. hyodysenteriae* strains. (A) PCR amplification of *cat* gene. Lane 1, molecular size markers (kb); lane 2, strain A203 (Cm^r Ty^r); lane 3, strain B204 (Cm^s Ty^r); lane 4, strain B78 (Cm^s Ty^s); lane 5, transductant strain CM-1 (Cm^r Ty^s). Seven other randomly selected transductant strains gave the same-size amplicon as CM-1 in the PCR assay. (B) DNA sequence analysis to detect *rrl* (23S rRNA gene) nucleotide change associated with tylosin (macrolide) resistance. Sequence 1 (Seq #1), strain B78 (Cm^s Ty^s); sequence 2, strain B204 (Cm^s Ty^r) (strain A203, strain Ty1, and five other randomly selected transductant strains had the same *rrl* sequence).

subinhibitory levels of antibiotics seems incongruous, it does occur. Antibiotic concentrations considered therapeutic for bacterial infections are subinhibitory for pathogenic bacteria with acquired resistance and for commensal species with acquired resistance or natural insensitivity to the antibiotic. Doxycycline is used at subantibacterial levels to reduce tissue destruction in periodontal disease (47). Animal feeds promoting weight gain/feeding efficiency contain antibiotic levels lower than those of feeds for disease therapy (14, 28). Explanations for the effectiveness of performance-enhancing medicated feeds are that they favor the growth/metabolism of insensitive, beneficial commensal species in the animal intestinal tract or prevent growth of subclinical sensitive pathogenic species (41).

From a genetic and evolutionary perspective, a noteworthy bacterial response to subinhibitory antibiotic exposure is the activation of mobile genetic elements carried by bacteria. These mobile elements include both chromosomally integrated conjugative elements and traditional prophages. The excision and transfer of SXT, a 100-kb chromosomally integrated conjugative element of *Vibrio cholerae* and a carrier of multiple-drug resistance and virulence-encoding genes, is activated by low levels of ciprofloxacin (5, 6). Tetracycline stimulates 100- to 1,000-fold the transfer of *Bacteroides* conjugative transposons carrying resistance to tetracycline and other antibiotics (48, 60).

Prophages of bacteria colonizing animals and humans often carry genes that are nonessential for the bacteriophage but that provide selective advantages for their bacterial host. Examples include genes encoding toxins and proteins affecting immune responses and antibiotic resistance (9, 26, 50). Stx (shiga toxin)-transducing prophages of *E. coli* have been induced by norfloxacin (40). Ciprofloxacin given in subtherapeutic doses increases gene transduction by *E. coli* Stx2 phages in the mouse gastrointestinal tract (61), although, interestingly, not in sheep rumens (16).

VSH-1 is the only known gene transfer mechanism of *B. hyodysenteriae*. The involvement of this GTA in the evolution of *B. hyodysenteriae* as a recombinant population has been proposed (57). VSH-1-like elements appear to be widely distributed among *Brachyspira* species, including strains in the human intestinal tract (11, 12, 30, 55). The evolutionary origins of VSH-1 are unclear, but the current and simplest explanation is that its ancestor was a fully functioning prophage (52).

Carbadox, a quinoxaline-di-*N*-oxide compound, and metronidazole, a 5-nitro-imidazole compound, are potent inducers of VSH-1. On a molar basis, they are 12 to 15 times more effective than mitomycin C at inducing *hvp38* transcription (Table 1).

Carbadox is an effective antimicrobial for preventing and treating intestinal diseases of postweaning swine, notably swine dysentery caused by *B. hyodysenteriae*. Carbadox is also used as a feed additive to promote swine growth (28, 43), although to our knowledge the basis for growth promotion is unknown. Although metronidazole and related 5-nitroimidazoles are no longer used for swine applications, they are used for treating anaerobic infections of nonfood animals (44). Nitroimidazole antibiotics are commonly used to treat human intestinal diseases caused by anaerobic bacteria and parasitic protozoa (4, 21, 45).

Under anaerobic conditions, carbadox and metronidazole are chemically reduced by bacterial metabolism to products that directly interact with bacterial DNA, causing mutations and DNA strand breaks (7, 51). Thus, an early event for VSH-1 induction by carbadox and metronidazole, and by mitomycin C and H_2O_2, is presumed to be DNA damage leading to a RecA-centered SOS response, as reported for other bacterial species (3, 5, 6, 10, 18, 32, 62).

Carbadox seems to be generally useful for inducing prophages from various bacterial species. Carbadox induces lambda-like prophages carrying shiga toxin (*stx*) genes in *E. coli* cultures (36) and is a component of a commercial test, EMD Duopath Verotoxins (Merck catalog no 1.04144.0001), to stimulate phage-associated Stx production (1). Recently, we and a colleague (S. Casjens, personal communication) have used 0.5 μg carbadox/ml of culture to induce prophages from *Shigella* and *Salmonella* cultures. In view of its low cost (approximately 10^{-6} the cost of a prophage-inducing amount of mitomycin C), carbadox is a more economical alternative to mitomycin C for bacteriophage or GTA induction.

Does carbadox in medicated feeds induce production of VSH-1, other GTAs, or traditional prophages by bacteria in the intestinal tracts of swine? The answer is not known. This is a question that is worth pursuing, in view of growing awareness that prophages play significant roles in bacterial evolution and ecology (8, 13). VSH-1 involvement in *B. hyodysenteriae* evolution has been proposed (57), and carbadox-induced VSH-1 particles transfer tylosin resistance between *B. hyodysenteriae* cells in culture (Table 2). Swine dysentery, or bloody scours, is associated with passage of blood through the intestinal mucosa at sites of *B. hyodysenteriae* colonization (34, 35). Swine feed contains 10 to 25 g carbadox/ton (11 to 28 mg/kg) for growth promotion and 50 g/ton (55 mg/kg) for disease prophylaxis (28). In a study of carbadox pharmacokinetics, swine fed 30 mg carbadox/kg of feed had 0.03 μg carbadox/ml blood (17). In *B. hyodysenteriae* cultures, VSH-1 is induced by carbadox at similar concentrations (Table 1 and Fig. 1).

The findings of these studies suggest a need to evaluate VSH-1 induction and lateral gene transfer in vivo as possible

collateral effects of carbadox medication. Additionally, GTA or prophage induction is a lethal event for the host bacterium and might contribute to the therapeutic or performance-enhancing properties of the antibiotic. Thus, a potentially broader impact of carbadox (and metronidazole) as a prophage inducer on intestinal microbial ecology in animals and humans deserves consideration.

ACKNOWLEDGMENT

Mention of trade names or commercial products in this article is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture.

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