Collateral Effects of Antibiotics: Carbadox and Metronidazole Induce VSH-1 and Facilitate Gene Transfer among \textit{Brachyspira hyodysenteriae} Strains\textsuperscript{V}

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\textit{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 \textit{B. hyodysenteriae} genome and transfer genes between \textit{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 \textit{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 \textit{hyp38} (VSH-1 head protein gene). VSH-1 induction was confirmed by detecting VSH-1-associated 7.5-kb DNA and VSH-1 particles in \textit{B. hyodysenteriae} cultures. Nine antibiotics (chlorotetracycline, lincomycin, tylosin, tiamulin, virginiamycin, ampicillin, ceftriaxone, vancomycin, and florfenicol) at concentrations affecting \textit{B. hyodysenteriae} growth did not induce VSH-1 production. By contrast, VSH-1 was detected in \textit{B. hyodysenteriae} cultures treated with mitomycin C (10 \textmu g/ml), carbadox (0.5 \textmu g/ml), metronidazole (0.5 \textmu g/ml), and \textit{H}$_2$\textit{O}$_2$ (300 \textmu M). Carbadox- and metronidazole-induced VSH-1 particles transmitted tylosin and chloramphenicol resistance determinants between \textit{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 \textit{Brachyspira hyodysenteriae}. Within their genome, \textit{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 \textit{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 \textit{B. hyodysenteriae} cells (31, 54). These unusual properties of VSH-1 are shared by similar elements in species of \textit{Rhodobacter}, \textit{Methanococcus}, and \textit{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. \textit{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 \textit{B. hyodysenteriae} cells.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The \textit{B. hyodysenteriae} strains B78\textsuperscript{T} (type strain), B204, and A203 were used in these studies. Strain B78\textsuperscript{T} is sensitive (MIC = 4 to 8 \textmu g/ml) to the macrolide antibiotic tylosin (33). Strain B204 is naturally resistant to tylosin (Ty\textsuperscript{r}) (MIC > 256 \textmu g/ml) and was derived from strain B204 by inserting a cat cassette into the \textit{flaA1} gene (31, 46).

\textit{B. hyodysenteriae} cells were routinely grown at 38°C in stirred 10-ml BHIS broth cultures beneath an initial N\textsubscript{2}-O\textsubscript{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 BHIS broth 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\textsubscript{620}] = 0.5 to 1.0; 18-mm path length).

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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 (OD620 = 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 2), metronidazole (0.005 to 2), chlorotetracycline (5 to 50), lincomycin (10 to 100), tylosin (500 to 1,000), tiamulin (0.02 to 2), virginiamycin (0.1 to 1.5), 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 four-fold 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). In some cases, RNA 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 (OD260) 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 GTGTTTGCTTCCATAAAGTTCTGCATCTGT. Based on the sequence (GenBank accession no. AY971355), the primers for amplifying 16S rRNA were 16F (5′-GGAGAGGATGATCGTATGCCAGG-3′) and 38R (3′-TATTCACACTTGTTGATCTAATTGA-5′). Amplification of 16S rRNA was carried out with the TaqMan One Step Master Mix reagent kit (Applied Biosystems) in a final volume of 25 μl.

Antibiotic concentrations affecting B. hyodysenteriae growth. B. hyodysenteriae B204 (Cms Tyr) and strain B78T (Cms Tys) in exponential growth phase (OD620 between 0.5 and 1) were cultured in BHIS broth (Fig. 1B). Amoxicillin (0.5 μg/ml) or lower were subinhibitory for B. hyodysenteriae growth. B. hyodysenteriae cells exposed to potential inducers of VSH-1 were examined by electron microscopy (Fig. 1A).

RESULTS

Antibiotics and growth inhibition. 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 OD620 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 H2O2 were found to impair or inhibit B. hyodysenteriae growth in BHIS broth (Fig. 1B). Among these,
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$_2$O$_2$ (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$_2$O$_2$-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$_2$O$_2$ 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

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Antimicrobial conc.</th>
<th>hvp38 transcription (fold increase)$^a$</th>
<th>VSH-1 production$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µg/ml</td>
<td>µM</td>
<td>DNA</td>
</tr>
<tr>
<td>Control (untreated)</td>
<td>1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Mitomycin C</td>
<td>10</td>
<td>30</td>
<td>260</td>
</tr>
<tr>
<td>Metronidazole</td>
<td>0.5</td>
<td>2.5</td>
<td>720</td>
</tr>
<tr>
<td></td>
<td>0.05</td>
<td>0.25</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>0.005</td>
<td>0.025</td>
<td>7</td>
</tr>
<tr>
<td>Carbadox</td>
<td>0.5</td>
<td>1.9</td>
<td>290</td>
</tr>
<tr>
<td></td>
<td>0.05</td>
<td>0.19</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>0.005</td>
<td>0.019</td>
<td>14</td>
</tr>
<tr>
<td>H$_2$O$_2$</td>
<td>300</td>
<td>100</td>
<td>100</td>
</tr>
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</table>

$^a$ 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).
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 (Tyr Cmr) cultures treated with carbadox or metronidazole. The VSH-1 particles were added to cultures of *B. hyodysenteriae* B204 (Ty/L Cm)*. 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 *A203* → *T* 23S rRNA gene base modification as the *B. hyodysenteriae* A203 strain from which VSH-1 had been induced (Fig. 4B).

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

<table>
<thead>
<tr>
<th>VSH-1 virion</th>
<th>No. of transductants/10⁸ CFU&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Tylosin resistant (10 µg/ml)</td>
</tr>
<tr>
<td>None (control)</td>
<td>&lt;LOD&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Mitomycin C induced</td>
<td>470</td>
</tr>
<tr>
<td>Metronidazole induced</td>
<td>405</td>
</tr>
<tr>
<td>Carbadox induced</td>
<td>250</td>
</tr>
</tbody>
</table>

<sup>a</sup> VSH-1 virions were induced by treating NT cultures of *B. hyodysenteriae* strain A203 (Ty/L Cm)* 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/L Cm)* 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.

<sup>b</sup> LOD, limit of detection. Spontaneously occurring tylosin-resistant cells were too few for accurate estimates (i.e., 2 to 4 CFU/10⁸ CFU). Spontaneously occurring chloramphenicol-resistant cells were not detected.

**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 (0.05 µg/ml); lane 3, carbadox (0.1 µg/ml); lane 4, H₂O₂ (200 µM); lane 5, H₂O₂ (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.

**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...
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 H2O2, 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^-16 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.

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REFERENCES