THE IONOPHORE RESISTANCE OF RUMINAL BACTERIA AND ITS RELATIONSHIP TO OTHER FORMS OF ANTIBIOTIC RESISTANCE

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INTRODUCTION

The FDA approved the ionophore monensin as feed additive for cattle in 1976, and within a few years most feedlot cattle in the United States were being fed this antibiotic (Russell and Strobel, 1989). Feed efficiency improvements as large as 10% were documented (Goodrich et al., 1984), and in vitro studies and in vivo incubations showed that monensin was altering ruminal fermentation (Dinius et al. 1976; Richardson et al., 1976). Since this time two other ionophores have also been approved in the United States for use in cattle as feed additives (e.g. lasalocid and laidlomycin). Because ionophores are antibiotics, some groups have argued that they pose a public health threat and have called for a ban on their use. Indeed, the European Union has proposed a future (January 1, 2006) ban on the use of ionophores as growth promotants (European Commission Directorate-General XXIV, 1999). Some ruminal bacteria are highly resistant to ionophores (Russell and Strobel, 1989). The question then arises, does the ionophore resistance of ruminal bacteria confer an increased resistance to other classes of antibiotics?

GROWTH PROMOTION OF CATTLE

Goodrich et al. (1984) reviewed the effects of monensin on the performance of nearly 16,000 feedlot cattle and reported that treated cattle gained weight 1.6% faster, consumed 6.4% less feed and had a 7.5% greater feed efficiency than controls. Grazing cattle (24 trials) fed monensin also gained weight 13.5% faster. Because monensin can modulate feed intake, it can increase the ruminal pH of cattle fed large amounts of grain (Nagaraja et al., 1982). Cattle fed legumes or ample amounts of starch can bloat, and some ionophores (e.g. monensin) have been effective in alleviating this disorder (Nagaraja et al., 1997).

EFFECTS ON RUMINAL FERMENTATION END-PRODUCTS

Monensin was originally marketed as a treatment to decrease methane production in the rumen, and this effect is mediated by its ability to inhibit bacteria that produce hydrogen, a precursor of methane (Van Nevel and Demeyer, 1977). When hydrogen production decreases, the bacteria must use alternative mechanisms to dispose of reducing equivalents (e.g. propionate production), resulting in an increase in energy retention from feed. The ability of monensin to increase the propionate to acetate ratio, however, only explains approximately 1/3 of the increase in efficiency (Russell and Strobel, 1989). Early work showed that monensin could decrease ammonia, a wasteful
end product of bacterial protein degradation (Richardson et al., 1976). Protein degradation is most important in cattle fed forages, but it may also be a factor in feedlot cattle. When feedlot cattle were treated with monensin, the response was greater if rations contained soybean meal rather than urea as a supplement (Lana et al., 1997). Furthermore, some lactic acid-producing bacteria are sensitive to monensin and this effect may contribute to its ability to prevent acidosis (Newbold and Wallace, 1988).

Effects of Monensin on Ruminal Fermentation

<table>
<thead>
<tr>
<th>Inhibition of Hydrogen Producers</th>
<th>Inhibition of Ammonia Producers</th>
<th>Inhibition of Lactate Producers</th>
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<tr>
<td>Less Methane</td>
<td>Less Ammonia</td>
<td>Less Lactate</td>
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<tr>
<td>Higher Propionate to Acetate</td>
<td>Greater Protein Availability</td>
<td>Higher pH</td>
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</tbody>
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Figure 1. The effect of the ionophore, monensin, on various aspects of ruminal fermentation. Taken with permission from J.B. Russell 2002. Rumen Microbiology and its Role in Ruminant Nutrition, p. 88. J. B. Russell Publishing Co., Ithaca New York.

IONOPHORE ACTIVITIES

Ionophores are highly lipophilic polyethers that accumulate in cell membranes and catalyze rapid ion movement (Pressman, 1976). Ionophores act as antiporters by binding to protons or metal ions (e.g. sodium, potassium, calcium and magnesium) and moving them across cell membranes. Because cell membranes are hydrophobic, only uncharged ionophores that are protonated or coordinated with a metal ion can move freely through them. Metal ion binding is facilitated by a loss of solvation water and the ability of the linear ionophore molecule to surround the ion and shield its charge (Riddell, 2002). Ionophores such as monensin and lasalocid are often more effective when the pH is low because their carboxyl group is more likely to be protonated (Chow and Russell, 1990). If the pH is higher than the pKₐ of the molecule, the ionophore is ionized, and this diminishes its ability to dissipate ion gradients. However, if the pH is below its pKₐ, the ionophore is protonated and can penetrate the cell membrane, release its proton, pick up a metal ion, and travel back across the cell membrane to catalyze a futile ion cycle (Figure 2). Monensin has a pKₐ of 7.95 and lasalocid has a pKₐ of 5.8 (Pressman, 1973).
The ability of an ionophore (I) to translocate protons (H⁺) and metal ions (M⁺) across the cell membrane of sensitive bacteria. The cells then use ATPases and transporters to counteract this flux. Taken with permission from J.B. Russell 2002. Rumen Microbiology and its Role in Ruminant Nutrition, p. 89. J. B. Russell Publishing Co., Ithaca New York.

The magnitude of ion gradients across the cell membrane dictates the direction of metal and proton movement by ionophores (Russell and Strobel, 1989). All living organisms maintain a higher concentration of potassium inside than outside their cells, and expel sodium and protons. When glucose-energized Streptococcus bovis cells are treated with monensin, intracellular potassium declines and there is an influx of sodium and protons. The cells then attempt to counteract futile ion flux by activating membrane ATPases and transporters, but they are eventually de-energized.

Ionophores can also translocate ions across the cell membranes of eukaryotes, and this characteristic limits their therapeutic use (Pressman, 1976). Some animals can tolerate ionophore doses needed to inhibit sensitive bacteria, but this tolerance is highly species specific. Horses are susceptible to relatively small doses of lasalocid and monensin, and these ionophores cause depression, ataxia, paresis, paralysis, anorexia, and death (Hanson et al., 1981). Cattle have liver enzymes that degrade ionophores and enterohepatic circulation recycles absorbed ionophore back to the gut via the bile (Donoho, 1984). The effect of ionophores on humans has not been experimentally determined. However, people exposed to monensin during its manufacture had symptoms including headache, nausea, nose bleed and skin rash, and ranchers that fed monensin to cattle had headache and dizziness (Pressman, 1985).
IONOPHORE RESISTANCE

Many monensin-resistant ruminal bacteria have the membrane-bound enzyme, fumarate reductase (Russell and Strobel, 1989). Bergen and Bates (1984) hypothesized that this proton-translocating enzyme might counteract ionophore-dependent ion flux, and Morehead and Dawson (1992) noted that monensin-resistant Prevotella ruminicola strains appeared to have more fumarate reductase activity than those that were monensin-sensitive. However, at least one fumarate reductase containing ruminal bacterium (Ruminococcus flavefaciens) is highly sensitive to monensin, and this observation did not support the fumarate reductase model of ionophore resistance (Chen and Wolin, 1979).

Ionophore resistance is more easily correlated with differences in the cell envelope structure (Russell and Strobel, 1989). Gram-positive bacteria that lack an outer membrane are, in many cases, more sensitive to monensin than Gram-negative species, and Gram-positives are more apt to produce acetate, butyrate, hydrogen and ammonia (negative byproducts of ruminal fermentation). The cell wall model of monensin-resistance is, however, not always straightforward. Some Gram-negative ruminal bacteria are ionophore-sensitive and need a period of adaptation before they can become resistant, and some Gram-positive species (e.g. S. bovis) are more resistant than Gram-negative bacteria (Chen and Wolin, 1979, Callaway et al., 1999, Callaway and Russell, 2000).

Prevotella bryantii is a Gram-negative ruminal bacterium, but a comparison of 15 Prevotella strains indicates that only some were highly resistant (Callaway and Russell, 2000). B14 was the most resistant strain, and potassium efflux measurements indicated that monensin-adapted cultures were 16-fold more resistant than those that were not exposed to sub-lethal doses of monensin (Callaway and Russell, 1999). Subsequent work indicated that the original culture had a mixture of monensin-sensitive and monensin-resistant cells (Callaway and Russell, 1999). Monensin treatment increased the number of cells in the population with the resistance phenotype, but sensitive cells dominated after monensin was withdrawn. Clostridium aminophilum is a Gram-positive, amino acid-fermenting ruminal bacterium that can be inhibited by relatively small doses of monensin in vitro (e.g. 1 μM), but can adapt and grow rapidly with this concentration of ionophore (Rychlik and Russell, 2002). This ability to adapt is consistent with the observation that physiological doses of monensin could not eliminate C. aminophilum from the rumen (Krause and Russell, 1996).

Recent research indicates that extracellular polysaccharides play a key role in the ionophore resistance of ruminal bacteria. When P. bryantii B14 (Callaway and Russell, 1999) and C. aminophilum F (Rychlik and Russell, 2002) cultures were adapted with monensin, the monensin-resistant cells were: 1) more easily dispersed, 2) had more exopolysaccharide, and 3) were not agglutinated by lysozyme, a positively charged protein. The resistant cells did not persist after the ionophore was withdrawn, and there was little indication that resistance was mediated by traditional mechanisms (e.g., a degradative enzyme or a pump that expelled antibiotic).
Figure 3. The ability of lysozyme, a positively charged protein, to agglutinate wild-type (a, c) and monensin-adapted (b, d) *P. bryantii* B₁₄ cells. Taken with permission from Callaway and Russell (Callaway and Russell, 1999).

![Image of lysozyme agglutination experiment]

Figure 4. The effect of monensin treatment (during the arrow) on the amount of monensin that is needed to cause 1/2 maximal potassium depletion from mixed ruminal bacteria. The cows were fed a diet consisting of timothy hay. Taken with permission from Lana and Russell (Lana et al., 1997).

![Graph showing the effect of monensin treatment on potassium depletion]
Because ionophores catalyze the efflux of potassium from sensitive cells, it is possible to monitor the resistance of mixed populations by measuring the amount of monensin needed to catalyze half maximal potassium depletion. When mixed ruminal bacteria were taken from cattle consuming hay, the potassium depletion constant was 0.2 µM, but this value increased 8-fold as soon as the cattle were supplemented with a daily dose of 350 mg monensin (Lana and Russell, 1996). Because the potassium efflux constant increased rapidly and returned to its original value as soon as monensin was withdrawn (Figure 4), it appeared that: 1) monensin resistance was prevalent before monensin is given, 2) increases in resistance occurred very rapidly, 3) the increased resistance did not persist if the ionophore was not present. These results are consistent with the idea that ionophores resistance is an adaptation rather than mutation or acquisition of foreign genes.

IONOPHORE RESISTANCE OF CLOSTRIDIUM AMINOPHILUM

The ionophore resistance of C. aminophilum F can be monitored by measuring either lag time in 1 µM ionophore or the ability to initiate growth in the presence of higher ionophore concentrations (>10 µM). Unadapted C. aminophilum F was initially sensitive to 1 µM monensin or lasalocid, however, rapid growth was eventually observed after long lag times (12 and 24 hours, respectively) (Figure 5).

![Figure 5](Image)

Figure 5. The growth of C. aminophilum F in basal broth without ionophore (■) and broth containing 1 µM monensin (△) or 1 µM lasalocid (□). The closed symbols show cultures that were transferred a second time with the same inhibitor. Redrawn from the data of Houlihan and Russell (2003).

The ionophore-adapted cultures did not lag a second time and were resistant to higher concentrations of ionophore. Non-adapted cultures could not initiate growth in 5 µM monensin or 2 µM lasalocid, but adapted cultures resisted monensin or lasalocid.
concentration as high as 10 μM. Resistant cultures that were transferred in basal broth lacking ionophore retained their resistance phenotype, but not for an indefinite period of time (4 to 9 transfers or 28 to 63 generations). This result indicated that ionophore resistance was not a stable trait.

When adapted C. aminophilum cultures were serially diluted into medium that contained 1 μM monensin, growth was observed at dilutions as high as $10^{-8}$, but growth was only observed in the $10^{-2}$ dilution if unadapted cultures were used. This result indicated that adapted cultures had 100,000 fold more resistant cells than non-adapted cultures, but subsequent work showed that virtually any cell could become resistant. If colonies (n = 10) from agar plates lacking ionophore were inoculated into broth containing 1 μM ionophore, growth was always observed. Based on this result it appears that ionophore-resistance is a physiological adaptation rather than the selection of a sub-population.

Chen and Wolin (1979) noted that Bacteroides (now Prevotella) ruminicola GA33 could become significantly more resistant to either monensin or lasalocid, but "there was no cross resistance of monensin mutants to lasalocid and vice versa." Monensin-adapted C. aminophilum cultures were resistant to 1 μM lasalocid, but lasalocid-adapted cultures lagged for 11 h when they were inoculated in broth containing 1 μM monensin. These results indicated that cross-resistance was possible, but the mechanism of resistance appears to be, in at least some cases, ionophore-specific.

**DISSEMINATION OF ANTIBiotic RESISTANCE**

Soon after the discovery that antibiotics could be used to treat human disease, it became apparent that some bacteria could acquire resistance, and these resistances can be to spread from one bacterium to another on extrachromosomal elements called plasmids (Levy, 2002). Multiply drug-resistant bacteria are now common, and some groups have argued that the routine use of antibiotics in animal feed creates a selective pressure for resistances that eventually spread to human pathogens (Levy, 2002). Agriculturists have argued that resistance is more apt to appear when physicians and veterinarians misdiagnose infection and improperly administer antibiotics. When antibiotics are misused in this fashion, the dosage is high, and the environment already has a large population of pathogens (e.g. hospitals).

The argument that antibiotics should not be used in animal feed was greatly strengthened by the observation that avoparcin use led in an increase in vancomycin resistance (Aarestrup, 1995). Avoparcin is a glycopeptide that was used to promote the growth of pigs and chickens in Europe (Bager et al., 1997). However, avoparcin is an analog of vancomycin, and this latter antibiotic is an important therapy for treating bacterial infections (Levy, 2002). Suspicions of cross-resistance arose when Klare et al. (1995) and Aarestrup (1995) isolated vancomycin-resistant enterococci from pigs and poultry, and Bager et al. (1997) confirmed the correlation between vancomycin-resistant Enterococcus faecium and avoparcin use. The European Union banned avoparcin’s use as a feed additive in 1997 (European Commission Directorate-General XXIV, 1999).
The extrapolation of avoparcin use to ionophores is, however, not well supported: 1) ionophores have never been (nor are likely to be) used as an antimicrobial for humans, 2) ionophores have a distinctly different mode of action from therapeutic antibiotics, and 3) ionophore resistance seems to be an adaptation rather than a mutation or acquisition of foreign genes. Because *C. aminophilum* F could be easily adapted to ionophores, we used this bacterium as a model to determine if ionophore resistance conferred an increased resistance to other classes of antibiotics. Results indicated that ionophore-resistant *C. aminophilum* F cultures were as susceptible to other classes of antibiotics (aminoglycosides, penicillin, vancomycin, rifampin, polypeptides, cephalosporin C, trimethoprim, lincomycin, tetracycline, erythromycin, chloramphenicol and novobiocin) as ionophore-sensitive ones (Figure 6). The only exception was bacitracin, an antibiotic only used in topical ointments because it is too toxic for systemic use.

![Figure 6. Effect of antibiotics on *C. aminophilum*. Open bars are unadapted cultures, solid bars were adapted with monensin, and shaded bars were adapted with lasalocid. Redrawn from the data of Houlihan and Russell (2003).](image)

When Aarestrup et al. (1998) examined various classes of gut bacteria (*Escherichia coli, Enterococcus faecalis, Enterococcus faecium, Campylobacter, Salmonella, Yersina, Staphylococcus, and Actinobacillus plero pneumoniae*) from Danish swine, monensin resistance was not frequently encountered, and Butaye et al. (2001) did not detect monensin-resistance in 146 *E. faecium* and 166 *E. faecalis* strains isolated from farm livestock and pet animals. These results support the idea that ionophore resistance is not easily spread from one bacterium to another, but similar work has not been performed in North America where animals have had a greater potential exposure to ionophores.
CONCLUSIONS

Only some animals can be safely fed ionophores, and ionophores have never been used as antibiotics for human therapy. Many ruminal bacteria are resistant to ionophores even if ionophores are not fed. However, there is little evidence that ionophore resistance confers an increased resistance to other classes of antibiotics or that ionophore resistance can be spread from one bacterium to another.

REFERENCES


