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The susceptibility of ionophore-resistant *Clostridium aminophilum* F to other antibiotics

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***Objective*: To determine if ionophore-resistant ruminal bacteria are cross-resistant to other classes of antibiotics. *Clostridium aminophilum* was used as a model organism because this Gram-positive ruminal bacterium can adapt to ionophores (monensin and lasalocid). Non-adapted cultures lagged for at least 12 h with 1 μM monensin or lasalocid, but initiated no growth if the concentration was 10 μM. Adapted cultures did not lag with 1 μM monensin or lasalocid, grew well even if the ionophore concentration was 10 μM and contained cells at least 100 000-fold more resistant than those in non-adapted cultures.**

***Methods*: Ionophore-adapted and non-adapted cultures were assayed for their susceptibility to other classes of antibiotics (penicillin G, ampicillin, cephalosporin C, vancomycin, carbenicillin, tetracycline, chloramphenicol, erythromycin, streptomycin, linocomycin, rifampicin, trimethoprim, novobiocin, polymyxin B and bacitracin) using a broth microdilution method.**

***Results*: Adapted cultures retained their resistance phenotype for at least 28 generations even if ionophore was no longer present. Monensin-adapted cultures were as resistant to lasalocid as those adapted to lasalocid, but lasalocid-adapted cultures lagged with 1 μM monensin. Monensin- and lasalocid-resistant *C. aminophilum* F cultures were as susceptible to most antibiotics as non-adapted cultures. The only antibiotic that seemed to have a common mechanism of resistance was bacitracin, and the ionophore-adapted cultures had a 32-fold greater MIC.**

***Conclusion*: The use of ionophores in cattle feed and the selection of ionophore-resistant ruminal bacteria does not necessarily lead to other types of antibiotic resistance.**

Keywords: antibiotics, ionophores, resistance, monensin, lasalocid

Introduction

In recent years, there has been concern regarding antibiotic use in agriculture and the spread of antibiotic resistance.[1] This concern was bolstered by the observation that avoparcin, an antibiotic used in Europe to promote livestock growth, led to an increase in vancomycin resistance.[2] The EU has proposed banning all antibiotics from livestock feed by 1 January 2006.[1] This ban includes the ionophores, a class of antibiotics that have never been used therapeutically. Ionophores were used originally to prevent coccidiosis in livestock, and are still used in this capacity throughout the world. Ionophores also decrease ruminal methane and ammonia production and improve the feed efficiency of cattle by as much as 10%.[3] Ionophores have never been used extensively for cattle in Europe, but are fed routinely to beef cattle in the USA.[4]

Ionophores have a distinctly different mode of action from therapeutic antibiotics,[5] and can be placed into three main classes: carboxylic polyether ionophores, neutral ionophores and pore-forming ionophores.[6] The polyether ionophores, which are used as feed additives for cattle, act as metal–proton antiporters.[5] By binding and shielding monovalent or divalent metal ions within a hydrophobic matrix, polyether ionophores can shuttle them across cell membranes.[7] Monensin translocates monovalent metal ions (e.g. sodium and potassium), but lasalocid can also bind divalent cations (e.g. magnesium and calcium).[7] The resulting electroneutral dissipation of ion and proton gradients leads to greater membrane ATPase and transporter activity. As a result, energy is diverted from growth to non-growth functions, and eventually susceptible cells are de-energized.[4]

Bacteria and mammalian enzymes can degrade ionophores, but these pathways are oxygen-dependent and not functional in anaerobic environments, such as the rumen or lower gastrointestinal tract.[8] Early work with ruminal bacteria showed that Gram-negative species

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were often less susceptible to ionophores than Gram-positive bacteria,[4] and this observation suggested that ionophore sensitivity and resistance are determined by the composition of the bacterial cell wall.

However, the cell wall model of ionophore resistance is not always straightforward. Some Gram-negative species need a period of adaptation before they can grow in the presence of ionophore.[9,10] Conversely, some Gram-positive species are as resistant to ionophores as many Gram-negative bacteria.[11,12] *Clostridium aminophilum* F is a Gram-positive, hyperammonia producing ruminal bacterium[13,14] that contributes to wasteful ruminal amino acid degradation.[15] *C. aminophilum* F can be inhibited by the ionophore monensin *in vitro*,[13] but work with 16S rRNA probes showed that physiological doses of monensin do not eliminate this bacterium from the rumen.[16] Recent work has demonstrated that *C. aminophilum* F cultures can be adapted with sublethal concentrations of monensin.[17]

The following experiments sought to: (1) define more precisely the ionophore resistance of *C. aminophilum* F, (2) determine if resistance to one ionophore confers cross-resistance to other ionophores, and (3) monitor the susceptibility of ionophore-resistant strains to other classes of antibiotics.

Materials and methods

Culture conditions

C. aminophilum strain F was grown anaerobically in a basal broth containing (per L): 292 mg K_2HPO_4, 292 mg KH_2PO_4, 480 mg $(NH_4)_2SO_4$, 268 mg NH_4Cl_2, 480 mg NaCl, 100 mg $MgSO_4.7H_2O$, 64 mg $CaCl_2.2H_2O$, 600 mg cysteine hydrochloride, 4 g Na_2CO_3, 15 g Casaminoacids (Difco Laboratories, Detroit, MI, USA) and 15 g yeast extract (Difco). The culture broth was prepared under O_2-free CO_2 and dispensed into tubes (18 × 150 mm) that were sealed with butyl rubber stoppers. Cultures were maintained in basal broth and incubated at 39°C for 12–48 h in a water bath. Ionophore-adapted cultures were kept in basal broth supplemented with 1 μM monensin or lasalocid. Samples were withdrawn from the culture tubes with a sterile hypodermic syringe, and growth was assessed from the increase in optical density (1 cm cuvette, 600 nm).

Cultures were also grown anaerobically in a glove box (Coy Laboratory Products, Ann Arbor, MI, USA). Agar plates (1.5% w/v) containing basal medium were spread with 100 μL of stationary phase culture that had been diluted 100 000 fold with sterile basal broth. The plates were incubated at 39°C for 48 h. Colonies were then transferred to basal medium with or without 1 μM monensin or lasalocid.

Growth with ionophore

Non-adapted *C. aminophilum* F cells were inoculated (1% v/v) into basal broth supplemented with either 1 μM monensin or lasalocid and incubated at 39°C for 48 h. Adapted cultures were then re-inoculated (1% v/v) into basal broth containing 1 μM monensin or lasalocid, and growth was monitored. Growth was assessed from the increase in optical density (1 cm cuvette, 600 nm).

Adapted and non-adapted cells were also inoculated (1% v/v) into basal broth supplemented with different amounts of monensin or lasalocid (0.6, 1, 2, 5 and 10 μM) and incubated at 39°C for 72 h. Growth was assessed from the increase in optical density (1 cm cuvette, 600 nm).

Enumeration

Stationary phase ionophore-adapted and non-adapted *C. aminophilum* F cultures were serially diluted (10-fold increments) into basal medium supplemented with 1 μM monensin or lasalocid to determine viable cell number. The dilution tubes were incubated at 39°C for 96 h, and growth was assessed from the increase in optical density (1 cm cuvette, 600 nm).

Antibiotics

All antibiotics were prepared anaerobically under O_2-free N_2 and added to bacterial cultures with a sterile hypodermic syringe. Monensin, lasalocid and erythromycin were dissolved in sterile 95% ethanol. Chloramphenicol, trimethoprim, rifampicin and tetracycline were dissolved in a 50:50 mixture of water and ethanol. Vancomycin, bacitracin, penicillin G, kanamycin, polymyxin B, cephalosporin C (a parent compound used to make other semisynthetic antibiotics), carbenicillin, lincomycin, streptomycin and ampicillin were dissolved in water. The antibiotic stock solutions were then sterile filtered (0.2 μm). The final concentration of ethanol in culture tubes was never >2% (v/v), and preliminary experiments indicated that this concentration of ethanol did not affect the growth of *C. aminophilum* F. All antibiotics were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

Susceptibility testing

Antibiotic susceptibility testing was performed using a broth microdilution method, adhering as closely as possible to the recommendation of the NCCLS.[18] The test medium was basal broth, and final concentrations of the antibiotics were in the range 0.004–1024 mg/L. The *C. aminophilum* F inoculum (100 μL, 5×10^5 cfu/mL) was added to 96-well microtitre plates containing antibiotics that had been diluted in two-fold increments. The plates were incubated anaerobically (35°C, 24 h), and the optical density of each well was determined (600 nm). The MIC was defined as the lowest concentration of antibiotic under which bacterial growth was no longer detectable.

Experimental design

All experimental determinations were performed in triplicate, and the mean, S.D. and coefficient of variation were computed. If the coefficient of variation was <10% and the difference among treatment means was large, statistics were not reported. When the data did not meet these criteria, statistical significance was assessed by the use of a Student's *t*-test.[19]

Results

C. aminophilum F cultures (1% inoculum) did not immediately grow in basal broth that contained 1 μM monensin (0.7 mg/L) (Figure 1). However, after a long lag time, the cultures eventually initiated rapid growth and reached approximately the same cell density as those grown in the absence of ionophore. Monensin-treated cells that were re-inoculated into basal broth containing 1 μM monensin did not lag a second time. A similar adaptation to 1 μM lasalocid (0.6 mg/L) was observed, but in this case, the initial lag period was considerably longer (24 versus 12 h).

Cultures that had not been exposed to ionophore grew in basal medium that contained either 5 μM monensin (3.5 mg/L) or 2 μM lasalocid (1.2 mg/L), but the optical densities were noticeably lower than untreated cultures even if the incubation period was 72 h (Figure 2). Non-adapted cultures that were treated with 10 μM monensin (7.0 mg/L) or 5 μM lasalocid (3.1 mg/L) were no longer viable. Cultures adapted with 1 μM ionophore resisted monensin or lasalocid concentrations as high as 10 μM, and the cell densities were similar to untreated cultures.

Monensin-resistant *C. aminophilum* F cultures that were inoculated into basal medium containing 1 μM lasalocid did not lag and

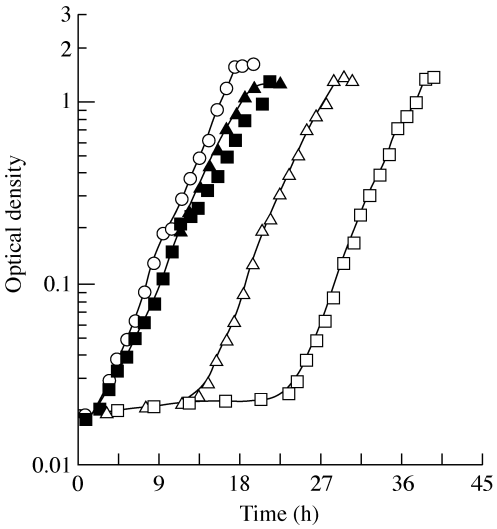

Figure 1. The growth of *C. aminophilum* F in basal broth without ionophore (open circles), in broth containing 1 μM monensin (open triangles) or 1 μM lasalocid (open squares). The solid symbols show cultures that were re-inoculated a second time with the same inhibitor.

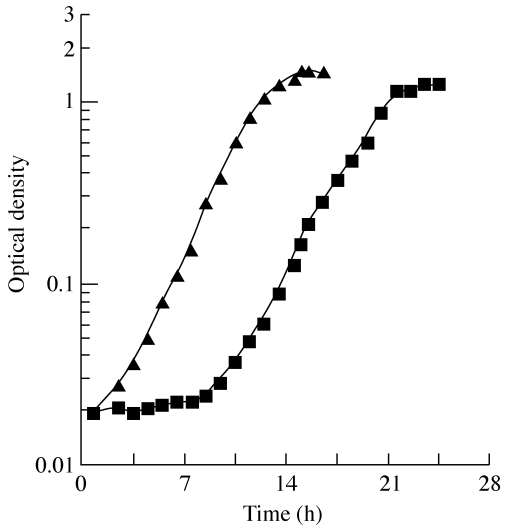

Figure 3. The growth of monensin-resistant *C. aminophilum* F in broth containing 1 μM lasalocid (triangles), and the growth of lasalocid-resistant *C. aminophilum* F in broth containing 1 μM monensin (squares).

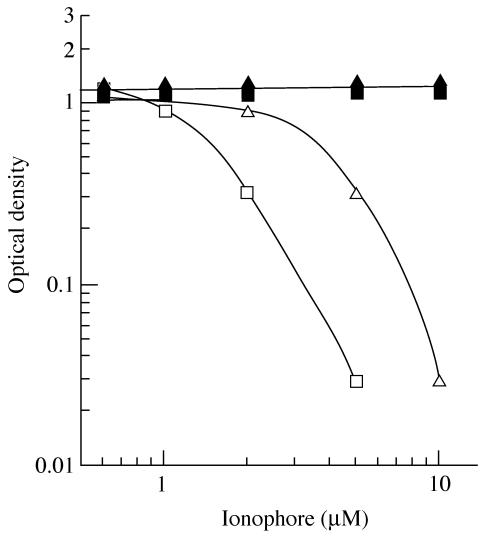

Figure 2. The effect of increasing amounts of monensin (triangles) or lasalocid (squares) on the growth of *C. aminophilum* F. The ionophore concentration is expressed on a log scale. Open symbols show cultures that were not adapted previously. Solid symbols show cultures that were adapted with 1 μM of the same ionophore.

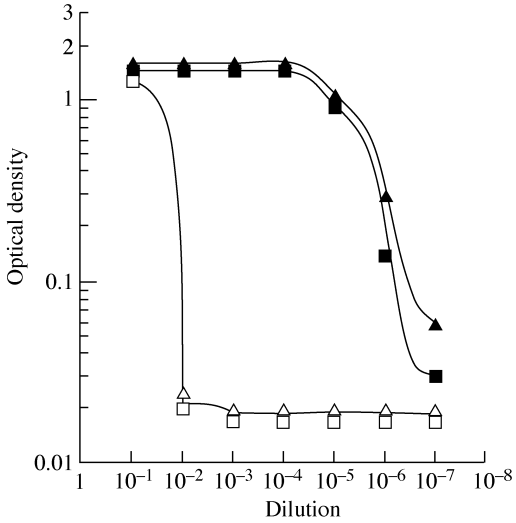

Figure 4. The growth of *C. aminophilum* F cultures that were serially diluted into broth containing 1 μM monensin (triangles) or 1 μM lasalocid (squares). Open symbols show cultures that were not previously adapted. Solid symbols show cultures that were adapted with 1 μM of the same ionophore.

grew rapidly, but the converse was not observed (Figure 3). If lasalocid-resistant cultures were treated with 1 μM monensin, an 11 h lag was observed before rapid growth. Monensin-resistant cultures that were subcultured every 24 h in broth lacking ionophore retained their resistance phenotype (ability to grow without lag in 1 μM monensin or the ability to grow in medium containing 10 μM monensin) for four subcultures (28 generations). Lasalocid-resistant cultures retained their phenotype for nine subcultures (63 generations).

Serial dilutions (10-fold increments) into basal broth containing 1 μM ionophore indicated that monensin- and lasalocid-adapted cultures had cells 100 000-fold more resistant than non-adapted cultures (Figure 4), but subsequent work indicated that virtually any cell could become resistant. If colonies (n = 10) taken from agar plates lacking ionophore were inoculated into broth containing 1 μM ionophore, growth was always observed. Because these cultures retained their ionophore resistance for several subcultures in the absence of ionophore, it was possible to determine the effect of ionophore resistance on susceptibility to other antibiotics without an antibiotic interaction (synergism) interfering with the results.

C. aminophilum F was naturally resistant to streptomycin, kanamycin, trimethoprim, and polymyxin B with MIC values of 128 mg/L (Table 1). The MIC values for cephalosporin C, tetracycline and baci-

Table 1. MICs for non-adapted, monensin-resistant and lasalocid-resistant *C. aminophilum* F cultures. All values are given in mg/L

Antibiotic	Target	Non-adapted	Monensin-resistant	Lasalocid-resistant
Penicillin G	cell wall	1	1	1
Ampicillin	cell wall	0.063	0.063	0.063
Cephalosporin C	cell wall	11	8	8
Vancomycin	cell wall	1	1	1
Carbenicillin	cell wall	0.5	0.5	0.5
Bacitracin	cell wall	4	128[a]	128[a]
Tetracycline	protein synthesis	9.33	8	8
Chloramphenicol	protein synthesis	0.167	0.125	0.125
Erythromycin	protein synthesis	0.031	0.031	0.031
Streptomycin	protein synthesis	128	128	128
Lincomycin	protein synthesis	0.016	0.016	0.016
Kanamycin	protein synthesis	128	128	128
Rifampicin	nucleic acid synthesis	0.008	0.008	0.008
Trimethoprim	nucleic acid synthesis	128	128	128
Novobiocin	nucleic acid synthesis	0.008	0.008	0.008
Polymyxin B	cell membrane	128	128	128

[a]Statistically significant ($P < 0.05$).

tracin were in the range 4–11 mg/L, and non-adapted *C. aminophilum* F cultures were sensitive to penicillin G, ampicillin, vancomycin, carbenicillin, chloramphenicol, erythromycin, lincomycin, rifampicin and novobiocin (MIC < 1 mg/L). Monensin- and lasalocid-resistant cultures had the same susceptibility to most classes of antibiotic as non-adapted cultures, but the ionophore-resistant cultures had greater MIC values for bacitracin.

Discussion

C. aminophilum F was isolated from a dairy cow not receiving ionophores, and it was described originally as a monensin-susceptible bacterium.[13] However, later work showed that it was only inhibited if the monensin concentration was significantly >1 μM.[16] Based on a rumen volume of 70 L and a daily intake of 350 mg, the concentration of monensin *in vivo* could be as high as 7 μM, but these simple calculations do not consider: (1) that the rumen operates as a continous culture system, (2) the pharmacokinetic parameters of monensin, (3) that ionophore-resistant bacteria can also bind monensin, and (4) that monensin can bind non-selectively to feed particles, protozoa and fungi.[20]

Monensin is the most commonly used ionophore in the cattle industry in the USA.[4] When cattle were fed a typical dose of monensin (350 mg/day), the amount of monensin needed to cause half maximal potassium efflux from mixed ruminal bacteria increased from 0.18 to 1.5 μM, and this change occurred in less than 3 days.[21] If cattle were fed 350 mg lasalocid per day, the amount needed to cause half maximal potassium efflux from mixed ruminal bacteria increased from 0.14 to 0.28 μM.[21] These data suggest that the bacterial population of the rumen can adapt rapidly and become resilient to these growth-promoting antibiotics.

Previous work indicated that monensin-treated *C. aminophilum* F cultures had a long lag time before they could adapt and grow,[17] and our experiments show that cultures treated with lasalocid had an even longer lag time. Because cultures treated with either 1 μM monensin or lasalocid did not lag a second time, it appeared that ionophore resistance was being selected. However, when individual colonies were picked from an agar plate lacking ionophore and transferred into broth containing ionophore 1 μM, growth was always observed. These results suggest that virtually any *C. aminophilum* F cell has the capacity to become resistant.

The NCCLS indicates that broth microdilution is the appropriate method for determining MIC for anaerobes, and we used this approach to assess the susceptibility of *C. aminophilum* F to other classes of antibiotics. However, culture conditions and pH, along with the ability of ionophores to interact with cell membranes, have a significant effect on their activity.[22] As a result, ionophore activity may not be determined accurately using MIC.

The ionophore resistance of *C. aminophilum* F could be assessed via lag time, or via the amount of ionophore that inhibited growth completely. Adapted cultures did not lag and tolerated much more ionophore than non-adapted cultures. In *Escherichia coli*, 'adaptive responses' are sometimes mediated by stationary-phase gene amplifications,[23] but the ionophore resistance of *C. aminophilum* was not a stable adaptation. Other workers have also noted that resistance factors are not always stable, but the nature of this instability has not been defined clearly.

Chen & Wolin[10] 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'. Butaye *et al.*[24] observed that enterococcal strains isolated from pigs and poultry fed ionophores exhibited cross resistance to salinomycin and narasin, but not monensin and lasalocid. Furthermore, resistance to monensin and lasalocid was not found. Our work shows that *C. aminophilum* F cultures adapted with monensin had an inherent increased resistance to lasalocid. However, growth of lasalocid-adapted cells lagged when they were treated with monensin. These results suggest that cross resistance is possible in *C. aminophilum* F, but the mechanism of resistance is, at least in some cases, ionophore-specific. Whether other classes of

microorganisms (e.g. coccidia) have a similar strategy of resistance has yet to be determined.

Therapeutic antibiotics have a variety of targets within bacterial cells (peptidoglycan synthesis, ribosome activity, DNA replication, mRNA transcription, nucleotide synthesis and membrane stability),[25] but ionophores have a distinctly different mechanism of action (ion translocation across the cell membrane).[5] Because only some animals can be fed ionophores safely,[8] this class of antibiotics never has been, and is unlikely to be, used as an antimicrobial for humans.[5] Therapeutic antibiotic resistance often involves degradative enzymes, efflux pumps, altered target sites or extracellular polysaccharide (glycocalyx) that serve as a diffusion barrier.[26–28]

The question then arises, would an ionophore-dependent increase in polysaccharide production lead to an increased resistance to other classes of antibiotics? Ionophores are highly hydrophobic molecules,[5] and previous work showed that monensin-resistant *C. aminophilum* F cultures had increased extracellular polysaccharide and were not agglutinized by lysozyme, a positively charged protein.[17] Based on these observations, we hypothesized initially that ionophore-resistant *C. aminophilum* F cultures would have increased resistance to therapeutic antibiotics, but there was little support for this hypothesis. The ionophore-resistant cultures were, in virtually all cases, as susceptible as non-adapted cultures. The only antibiotic with a greater MIC value was bacitracin, and work conducted with Gram-negative bacteria indicates that extracellular polysaccharides may play a role in bacitracin resistance.[29]

In 1999, the Scientific Steering Committee of the EU[1] stated that: 'regarding the use of antimicrobials as growth promoting agents, the use of agents from classes which are or may be used in human or veterinary medicine (i.e. where there is a risk of selecting for cross resistance to drugs used to treat bacterial infections) should be phased out as soon as possible and ultimately abolished'. However, there has been little evidence that ionophore resistance is becoming a problem. When Aarestrup *et al*.[30] examined indicator bacteria, zoonotic bacteria and animal pathogens from Danish food animals, monensin resistance was not encountered frequently. Butaye *et al*.[31] did not detect monensin-resistance in 146 *Enterococcus faecium* and 166 *Enterococcus faecalis* strains isolated from farm and pet animals in Europe. However, similar work has not been carried out in North America, where animals have had a greater potential exposure to ionophores. Results with *C. aminophilum* F are consistent with the idea that ionophore resistance is not necessarily associated with resistance to most other classes of antibiotics, but further work will be needed to confirm this idea.

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Mandatory disclaimer: 'Proprietary or brand names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by the USDA implies no approval of the product, and exclusion of others that may be suitable.'

References

1. European Commission Directorate-General XXIV. (1999). Consumer Policy and Consumer Health Protection, Directorate B—Scientific Health Opinions, Unit B3—Management of Scientific Committees II, Opinion of the Scientific Steering Committee on Antimicrobial Resistance, 28 May 1999.

2. Aarestrup, F. M. (1995). Occurrence of glycopeptide resistance among *Enterococcus faecium* isolates from conventional and ecological poultry farms. *Microbial Drug Resistance* **1**, 255–7.

3. Goodrich, R. D., Garrett, J. E., Gast, D. R. *et al.* (1984). Influence of monensin on the performance of cattle. *Journal of Animal Science* **58**, 1484–98.

4. Russell, J. B. & Strobel, H. J. (1989). The effect of ionophores on ruminal fermentation. *Applied Environmental Microbiology* **55**, 1–6.

5. Pressman, B. C. (1985). *Antibiotics and their Complexes* (Sigel, H., Ed.). Marcel Dekker, Inc., New York, NY, USA.

6. Butaye, P., Devriese, L. A. & Haesebrouck, F. (2003). Antimicrobial growth promoters used in animal feed: effects of less well known antibiotics on Gram-positive bacteria. *Clinical Microbiology Reviews* **16**, 175–88.

7. Westley, J. W. (1982). *Polyether Antibiotics*. Marcel Dekker, Inc., New York, NY, USA.

8. Donoho, A. L. (1984). Biochemical studies on the fate of monensin in animals and the environment. *Journal of Animal Science* **58**, 1528–39.

9. Callaway, T. R. & Russell, J. B. (1999). Selection of a highly monensin-resistant *Prevotella bryantii* sub-population with altered outer membrane characteristics. *Applied Environmental Microbiology* **65**, 4753–9.

10. Chen, M. & Wolin, M. J. (1979). Effect of monensin and lasalocid-sodium on the growth of methanogenic and rumen saccharolytic bacteria. *Applied Environmental Microbiology* **38**, 72–7.

11. Callaway, T. R., Adams, K. A. & Russell, J. B. (1999). The ability of 'low G+C Gram-positive' ruminal bacteria to resist monensin and counteract potassium depletion. *Current Microbiology* **39**, 225–30.

12. Callaway, T. R. & Russell, J. B. (2000). Variations in the ability of ruminal Gram-negative *Prevotella* species to resist monensin. *Current Microbiology* **40**, 185–90.

13. Chen, G. & Russell, J. B. (1989). More monensin-sensitive, ammonia producing bacteria from the rumen. *Applied Environmental Microbiology* **55**, 1052–7.

14. Paster, B., Russell, J. B., Yang, C. M. J. *et al.* (1993). Phylogeny of ammonia-producing ruminal bacteria, *Peptostreptococcus anaerobius*, *Clostridium sticklandii* and *Clostridium aminophilum* sp. nov. *International Journal of Systematic Bacteriology* **43**, 107–10.

15. Rychlik, J. L. & Russell, J. B. (2000). Mathematical estimations of hyper-ammonia producing ruminal bacteria and evidence for bacterial antagonism that decreases ruminal ammonia production. *FEMS Microbiology Ecology* **32**, 121–8.

16. Krause, D. O. & Russell, J. B. (1996). An rRNA approach for assessing the role of obligate amino acid-fermenting bacteria in ruminal amino acid degradation. *Applied Environmental Microbiology* **62**, 815–21.

17. Rychlik, J. L. & Russell, J. B. (2002). The adaptation and resistance of *Clostridium aminophilum* F to the butyrivibriocin-like substance of *Butyrivibrio fibrisolvens* JL5 and monensin. *FEMS Microbiology Letters* **209**, 93–8.

18. National Committee for Clinical Laboratory Standards. (2001). *Methods for Antimicrobial Susceptibility Testing of Anaerobic Bacteria—Fifth Edition: Approved Standard M11-A5*. NCCLS, Wayne, PA, USA.

19. Sokal, R. R. & Rolf, F. J. (1973). *Biometry*. W. H. Freeman, Co., San Francisco, CA, USA.

20. Chow, J. M., Van Kessel, J. A. S. & Russell, J. B. (1994). Binding of radiolabelled monensin and lasalocid to ruminal microorganisms and feed. *Journal of Animal Science* **72**, 1630–5.

21. Lana, R. P. & Russell, J. B. (1996). Use of potassium depletion to assess adaptation of ruminal bacteria to ionophores. *Applied Environmental Microbiology* **62**, 4499–4503.

22. Chow, J. M. & Russell, J. B. (1990). Effect of ionophores and pH on growth of *Streptococcus bovis* in batch and continuous culture. *Applied Environmental Microbiology* **56**, 1588–93.

23. Hastings, P. J. & Rosenberg, S. M. (2002). In pusuit of a molecular mechanism for adaptive gene amplification. *DNA Repair* **1**, 111–23.

24. Butaye, P., DeVriese, L. A. & Haesebrouck, F. (2000). Incomplete cross resistance against ionophores in *Enterococcus faecium* and *Enterococcus faecalis* strains from pigs and poultry. *Microbial Drug Resistance* **6**, 59–61.

25. Levinson, W. & Jawetz, E. (2000). *Medical Microbiology and Immunology.* Lange Medical Books/McGraw-Hill, New York, NY, USA.

26. Walsh, C. (2000). Molecular mechanisms that confer antibacterial drug resistance. *Nature* **406**, 775–81.

27. Costerton, J. W., Stewart, P. S. & Greenberg, E. P. (1999). Bacterial biofilms: a common cause of persistent infections. *Science* **284**, 1318–22.

28. Anderl, J. N., Franklin, M. J. & Stewart, P. S. (2000). Role of antibiotic penetration limitation in *Klebsiella pneumoniae* biofilm resistance to ampicillin and ciprofloxacin. *Antimicrobial Agents and Chemotherapy* **44**, 1818–24.

29. Pollock, T. G., Thorne, L., Yamazaki, M. *et al.* (1994). Mechanisms of bacitracin resistance in Gram-negative bacteria that synthesize exopolysaccharides. *Journal of Bacteriology* **176**, 6229–37.

30. Aarestrup, F. M., Bager, F., Jensen, N. S. *et al.* (1998). Surveillance of antimicrobial resistance in bacteria isolated from food animals to antimicrobial growth promoters and related therapeutic agents in Denmark. *APMIS* **106**, 606–22.

31. Butaye, P., DeVriese, L. A. & Haesebrouck, F. (2001). Differences in antibiotic resistance patterns in *Enterococcus faecalis* and *Enterococcus faecium* strains isolated from farm and pet animals. *Antimicrobial Agents and Chemotherapy* **45**, 1374–8.