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The Bacteriocins of Ruminal Bacteria and Their Potential as an Alternative to Antibiotics

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Abstract

Beef cattle have been fed ionophores and other antibiotics for more than 20 years to decrease ruminal fermentation losses (e.g methane and ammonia) and increase feed efficiency, and these improvements have been explained by an inhibition of Gram-positive ruminal bacteria. Ionophores are not used to treat human disease, but there has been an increased perception that antibiotics should not be used as feed additives. Some bacteria produce small peptides (bacteriocins) that inhibit Gram-positive bacteria. In vitro experiments indicated that the bacteriocin, nisin, and the ionophore, monensin, had similar effects on ruminal fermentation. However, preliminary results indicated that mixed ruminal bacteria degraded nisin, and the ruminal bacterium, *Streptococcus bovis*, became highly nisin-resistant. A variety of ruminal bacteria produce bacteriocins, and bacteriocin production has, in some cases, been correlated with changes in ruminal ecology. Some ruminal bacteriocins are as potent as nisin in vitro, and resistance can be circumvented. Based on these results, ruminal bacteriocins may provide an alternative to antibiotics in cattle rations.

Introduction

Simple stomached animals (e.g. pigs, chickens, rats and man) lack enzymes that can degrade cellulose or hemicellulose, and fibrous materials are poorly utilized. Ruminant animals (e.g. cattle, sheep, goats, deer, etc.) do not synthesize fiber digesting enzymes, but they have formed a symbiotic relationship with ruminal microorganisms that can. The ruminant provides the microorganisms with a habitat for their growth, the rumen, and microorganisms supply the animal with fermentation acids, microbial protein and vitamins (Hungate, 1966). However, ruminal fermentation also produces methane and ammonia, and these end-products are a loss of energy and nitrogen, respectively. When methane is inhibited, acetate production declines, the fermentation is diverted towards propionate (Figure 1), and energy retention increases (Wolin, 1975). If proteins can be protected from ruminal deamination, ammonia declines and the animal has more amino acids for its nutrition (Leng and Nolan, 1983). Some ruminal bacteria produce lactic acid at a rapid rate, and this acid can cause pronounced declines in ruminal pH, founder, and in severe cases, even death of the animal (Owens *et al.*, 1998). Ruminant nutritionists, farmers and ranchers have used ionophores and other antibiotics to modify ruminal fermentation and increase the efficiency of feed digestion (Russell and Strobel, 1989). However, there has been an increased perception that antibiotics should not be routinely used as feed additives (Russell and Rychlik, 2001).

Manipulation of Ruminal Fermentation

The rumen is inhabited by bacteria, protozoa and fungi, but bacteria play a dominant role in all facets of ruminal fermentation (Hungate, 1966). Gram-positive bacteria produce more ammonia, hydrogen, and lactate than Gram-negative species, and compounds that inhibit Gram-positive ruminal bacteria have increased feed efficiency (Russell and Strobel, 1989). In the 1970's, the Food and Drug Administration approved monensin as a feed additive for beef cattle, and this antibiotic is primarily effective against Gram-positive bacteria (Russell and Strobel, 1989). When monensin (an ionophore) reaches the cell membrane, there is an electro-neutral exchange of protons for monovalent cations, intracellular potassium declines, sodium accumulates, and the cells are de-energized (Figure 2).

The outer membrane of Gram-negative ruminal bacteria can protect the cell membrane from monensin (Russell and Strobel, 1989), but some Gram-negative bacteria are sensitive to monensin (Chen and Wolin, 1979; Callaway and Russell, 1999). Conversely, Gram-positive ruminal bacteria can be more resistant to monensin than some Gram-negative species (Callaway *et al.*, 1999). The relationship between cell wall type and ionophore resistance is confounded by the fact that ruminal bacteria in vivo have a thick glycocalyx and stain Gram-variable (Hungate, 1966; Costerton *et al.*, 1974). 16S rDNA has been used to classify ruminal bacteria, but some ruminal bacteria have outer membranes even though they are most closely related to Gram-positive species (e.g. *Selenomonas ruminantium* and *Megasphaera elsdenii*) (Collins *et al.*, 1994).

Monensin has been fed to beef cattle for more than 20 years, and there is little indication that

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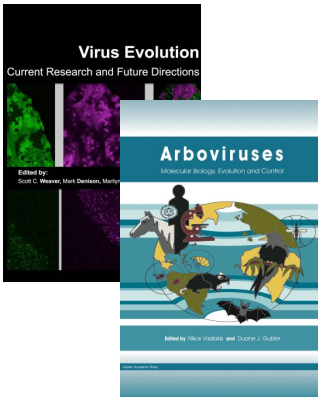

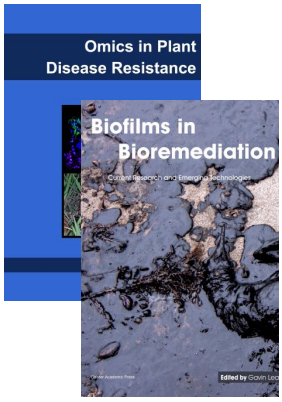

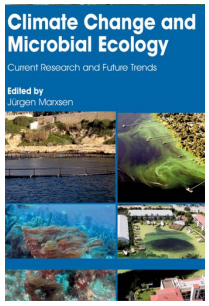

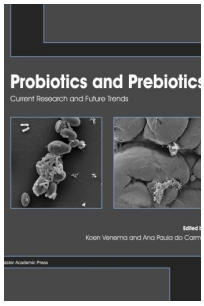

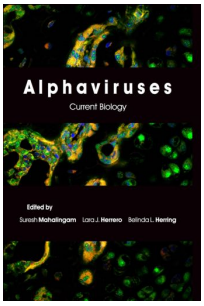

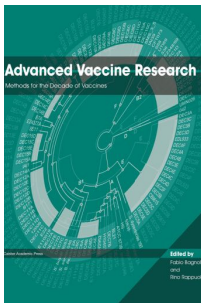

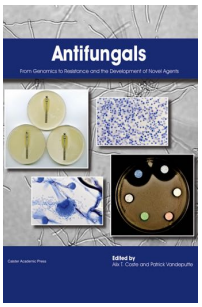

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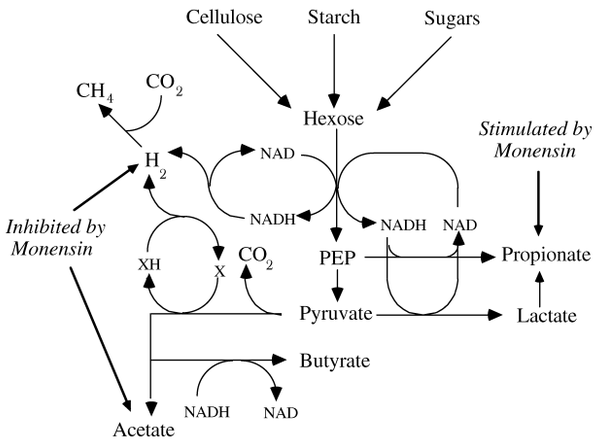

Figure 1. A schematic showing the fermentation schemes of ruminal bacteria. Interspecies hydrogen transfer and hydrogen utilization by methanogens are a primary means of reducing equivalent disposal. If gram-positive bacteria are inhibited, interspecies hydrogen transfer declines, and the fermentation shifts towards propionate, lactate and butyrate so dehydrogenase reactions can be used to oxidize the reducing equivalents.

ionophore resistance is counteracting the improvement in feed efficiency (Russell and Rychlik, 2001). Ionophores like monensin, however, can cause problems. Some livestock species (e.g. horses) are very sensitive to monensin, and doses recommended for cattle will kill them (Pressman and Fahim, 1982).

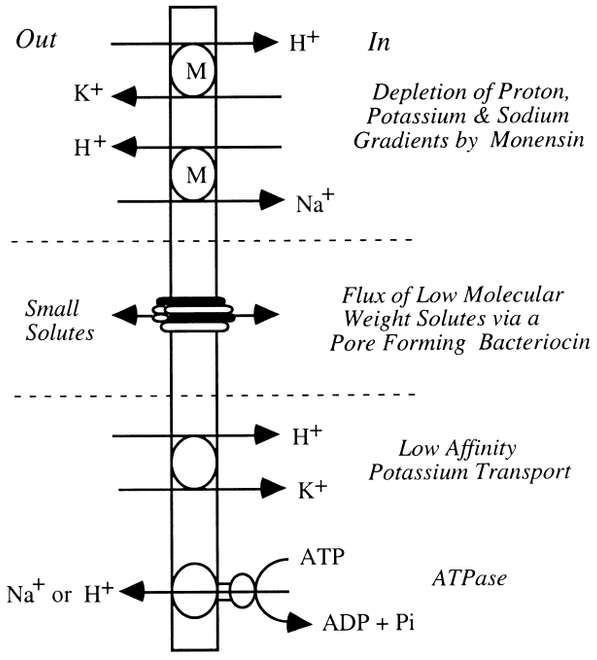

Figure 2. A schematic showing the effect of monensin (M) on the ion gradients of gram-positive bacteria. If the potassium, sodium and proton gradients are destroyed, the bacterium is forced to use low affinity potassium transport and ATPases to re-establish these gradients. Bacteriocins can also destroy these gradients but the specificity of this action is not great.

Toxicity is a serious problem if feed mills supply a variety of livestock species. Ionophores are not used as therapeutic agents by physicians (Teuber, 2001). However, humans exposed to monensin during the manufacturing process have reported symptoms that include headache, nausea, nosebleed and skin rash, and people feeding monensin to animals have had similar problems (Pressman and Fahim, 1982).

Bacteriocins

In 1925, Gratia observed that strains of *E. coli* could inhibit one another, and Rogers (1928) reported that lactococci could also produce antibacterial substances. Whitehead (1933) then demonstrated that the lactococcal factor was proteinaceous. Mattick and Hirsh (1944) tested the concentrated factor against pathogenic streptococci, and Taylor *et al.* (1949) attempted to use the same inhibitory substance to treat bovine mastitis. Lactococcal strains produce a variety of antibacterial substances (Hirsch and Grinsted, 1951), and these compounds were initially called ''antibiotics.'' However, the term ''bacteriocin'' was introduced in the 1950's to differentiate these ribosomally synthesized peptides from classical antibiotics (Jacob *et al.*, 1953). The classical definition of bacteriocins was largely based on colicins (Tagg *et al.*, 1976), and bacteriocins have been recently re-defined (Montville and Kaiser, 1993).

Mattick and Hirsh (1947) coined the term ''nisin'' to describe the group *N* inhibitory substance of *Lactococcus lactis*, and nisin has been the most studied and best understood bacteriocin (Jack *et al.*, 1995). Nisin is a relatively short peptide (34 amino acids) with five unusual sulfur-containing (lanthionine) rings and a number of dehydrated residues (Breukink *et al.*, 1998; Liu and Hansen, 1990). Nisin molecules appear to assemble in the cell membrane to form a barrel-like structure that facilitates the loss of intracellular solutes (Moll *et al.*, 1999). Early work suggested that barrel formation was dependent on a membrane potential (Moll *et al.*, 1999), but more recent experiments indicate that lipid II acts as an anchor in the cell membrane (Breukink *et al.*, 1999). Nisin also appears to inhibit the peptidoglycan synthesis of Gram-positive bacteria (Wiedemann *et al.*, 2001), but the independence of this activity from the dissipation of proton motive force has not been clearly established.

Lactococci that produce nisin are resistant to its activity, and this resistance is conferred by immunity proteins (Nis I, F, E and G) (Dodd *et al.*, 1996; Saris *et al.*, 1996). Nis I is a lipoprotein that binds to nisin in the outer surface of the cell membrane and prevents its insertion. The roles of Nis F, E and G are less well understood, but they appear to be specialized ABC exporters that expel free nisin from the cell or facilitate its intracellular degradation (Guder *et al.*, 2000; McAuliffe *et al.*, 2001; Saris *et al.*, 1996). The structural gene of nisin (*nis*A), *nis*I and *nis*FEG are part of a large operon that also includes genes for post-translational modification (*nis*B and *nis*C), a proteinase that hydrolyses the leader peptide (*nis*P) and the nisin transporter (*nis*T) (Guder

et al., 2000; de Vos *et al.*, 1995). The nisin gene cluster is located in a 70 kb transposon that also has the genes for sucrose utilization, and this transposon has been used as a tool for genetic studies (Klaenhammer *et al.*, 1993). Some strains carry the nisin operon as a chromosomal element, but other strains appear to have plasmid mediated nisin production and resistance (McKay and Baldwin, 1984; Tsai and Sandine, 1987; Horn *et al.*, 1991; Gireesh *et al.*, 1992).

Nisin has been a useful model of bacteriocin activity in Gram-positive bacteria, but many bacteriocins have distinctly different structures and spectrum of activity (Klaenhammer, 1993; Sablon *et al.*, 2000). Nisin is a class I lantibiotic, and this group is described as small, membrane-active peptides with unusual amino acids. Class II bacteriocins are also small (20–60 amino acids), positively charged peptides, but they do not contain lanthionine amino acids. Class III are large, heat-labile proteins. Class IV are complex bacteriocins, but their existence is ''questionable'' (Sablon *et al.*, 2000).

Class I bacteriocins are exported by specific ABC transporters, but some class II bacteriocins have a sec-dependent leader peptide and use the general secretory pathway (Worobo *et al.*, 1995; Cintas *et al.*, 1997; Kalmokoff *et al.*, 2001). Immunity to one type of bacteriocin does not usually confer immunity to another, and this characteristic enhances the ability of bacteriocin-producing bacteria to target specific populations. Nisin is active against a variety of Gram-positive bacteria, but many bacteriocins can only inhibit closely related strains or species.

Effect of Nisin on Ruminal Fermentation

When mixed ruminal bacteria were incubated in vitro with ground hay, even low concentrations of purified nisin inhibited methane production, decreased the acetate to propionate ratios and reduced ammonia production from a mixture of peptides and amino acids (Callaway *et al.*, 1997). More recently, Klieve and Hegarty (1999) suggested that bacteriocins could be used to decrease ruminal methane production in vivo. Because nisin seemed to be as potent an additive as monensin, a feeding trial was conducted to assess the ability of nisin to alter fermentation end-products. Previous work had shown that monensin (350 mg/day) could decrease the steady state concentration of ammonia in cattle (fed 12 times per day with a rotary feeder) by more than 50%, and this decline could be explained as a decrease in the specific activity of the mixed population to deaminate amino acids in vitro (Yang and Russell, 1993). Monensin also caused a significant (approximately 2-fold) decrease in acetate to propionate ratio. However, when cattle consuming the same diet were fed a similar amount of nisin, changes in ammonia concentration, the specific activity of deamination or acetate to propionate ratio could not be detected (Mantovani and Russell, unpublished results). These latter results suggested that nisin was either being degraded or the bacteria were becoming nisin-resistant.

Nisin Resistance and Degradation

Breukink *et al.* (1999) recently stated that ''no resistance to nisin has been reported'', but non-nisin producing bacteria that lack immunity proteins can become nisin-resistant. In *Listeria*, nisin-resistance has been correlated with a change in membrane lipids (Mazzotta and Montville, 1997). Recent work indicates that nisin sensitivity of *Staphylococcus aureus* can be increased by a change in teichoic acids, but the reverse (increased resistance) was not demonstrated (Peschel *et al.*, 1999).

When the ruminal bacterium, *S. bovis* JB1, was treated with nisin, growth was inhibited, but the nisin-treated cultures eventually grew as rapidly as untreated controls (Mantovani and Russell, 2001). Nisin initially caused a large decrease in viability, but cells that survived became nisin-resistant and retained this phenotype even if transferred many times in the absence of nisin. Based these results, it appeared that nisin was selecting for a sub-population of nisin-resistant cells, but subsequent experiments indicated that even sensitive cells could become resistant after only a short period of nisin exposure.

Rekhif *et al.* (1994) concluded that the bacteriocin resistance of *L. monocytogenes* was mediated by a high frequency mutation (as high as 10^{-3}), and the resistant cells had a stable phenotype. In *S. bovis*, the development of nisin resistance was also very quick, but the cells did not revert (Mantovani and Russell, 2001). Because the reversion rate was very slow, the cells remained nisin-resistant for long periods of time, even if nisin was not present. *S. bovis* cultures that were obtained directly from the rumen were initially as nisin-sensitive as the wild-type *S. bovis* JB1 cultures, but they also could develop nisin resistance (Mantovani and Russell, 2001). This result indicated that nisin-sensitivity was not simply an artifact of laboratory cultures, and conversely, nisin resistance does not appear to be an inherent property of naturally occurring *S. bovis* cells.

Viability (e.g. minimum inhibitory concentration) has often been used as an index of nisin sensitivity (and resistance), but viability alone gives little insight on the mechanism of action. Because nisin is a pore forming bacteriocin, potassium efflux can also be used to assess the effect of nisin on target bacteria. Experiments with *S. bovis* indicated that nisin-resistant cells did not lose as much potassium as sensitive cells, and this decline in potassium loss could be explained by a decreased binding of nisin to the cells (Mantovani and Russell, 2001).

Nisin is a positively charged molecule (Breukink and Kruijff, 1999), and nisin-resistant *S. bovis* cells were able to bind less cytochrome *c* than nisin-sensitive cells (Mantovani and Russell, 2001). The idea that *S. bovis* was excluding nisin was supported by the observation that nisin-resistant cells were less hydrophobic, were more lysozyme-resistant, and had more lipoteichoic acids than nisin-sensitive cells. When the de-esterified lipoteichoic acids were separated on polyacrylamide gels, extracts from nisin-resistant cells migrated more slowly than those from nisin-sensitive cells.

Work with *L. monocytogenes* indicated that nisin-resistant cells grew more slowly than sensitive ones (Mazzotta and Montville, 1997), but nisin-resistant and -sensitive *S. bovis* JB1 cultures had the same maximum growth rate (Mantovani and Russell, 2001). However, co-cultures indicated that the resistant cells only persisted if nisin was present (Mantovani and Russell, 2001).

Jarvis (1967) concluded that some nisin-resistant bacilli had ''nisin-inactivating enzymes'', but nisinases had not been purified, cloned or sequenced. When nisin-sensitive *S. bovis* cells were incubated with 1 μM nisin, there was an almost immediate and complete loss of intracellular potassium, but this loss could be prevented if the nisin was pre-incubated with clarified (10% v/v) ruminal fluid (Lee *et al.*, in press). Based on these results, it appears that mixed ruminal bacteria are able to degrade nisin.

Bacteriocins of Ruminal Bacteria

Streptococcus bovis

S. bovis is a rapidly growing and opportunistic low G + C bacterium that only becomes a dominant ruminal bacterium if the diet contains large amounts of soluble sugar or starch (Hungate, 1966; Owens *et al.*, 1998). Early work indicated that some *S. bovis* strains produced bacteriocins, but only one of these strains was isolated from the rumen (Iverson and Mills, 1976). More recently, Whitford *et al.* (2001a) screened 35 laboratory cultures, and they noted that approximately 20% of the *S. bovis* inhibited other streptococci. When fresh isolates from cattle fed hay or grain were overlayed with agar containing *S. bovis* JB1, approximately 50% of the *S. bovis* strains produced a zone of clearing (Mantovani *et al.*, 2001). These results indicated that bacteriocin production was a common feature of *S. bovis*, but bacteriocin producing strains did not displace sensitive ones in vivo.

S. bovis strains have similar 16S rRNA sequences (Klieve *et al.*, 1999), and 16S rRNA polymorphism is not a useful tool for comparing individual strains (Jarvis *et al.*, 2000). However, when repetitive DNA sequences (BOX elements) of freshly isolated *S. bovis* were amplified, a variety of PCR products were observed, the DNA fingerprints could be compared, and the strains were organized into 16 BOX types (Mantovani *et al.*, 2001). Diet (hay versus grain) had little impact on BOX type, and bacteriocin production was not a genetically conserved trait.

Whitford *et al.* (2001a) purified a bacteriocin from a bacterium originally thought to be *S. bovis,* but this isolate came from a moose and was more closely related to *Streptococcus gallolyticus* LRC0255 than *S. bovis* ATCC 33317 (the type strain). The bacteriocin of LRC0255 (bovicin 255) is a positively charged molecule, and recent work indicated that bovicin 255 could inhibit nisin-sensitive *S. bovis* but not nisin-resistant cells (Mantovani *et al.*, 2001). When 90 freshly isolated *S. bovis* were serially diluted into sterile filtered culture supernatant from *S. gallolyticus* LRC0255, there was only a small decrease in viable cell number (1.8 ± 2.1 log cells/ml), and the bovicin-sensitive strains adapted.

16S rDNA indicated that a freshly isolated strain designated as HC5 was closely related to other *S. bovis*, and this strain produced a bacteriocin-like substance that could inhibit nisin-sensitive and nisin-resistant *S. bovis* JB1 (Mantovani *et al.*, 2001). When 90 freshly isolated *S. bovis* strains were serially diluted into sterile filtered HC5 supernatant, the average decrease in viability was 4.8 ± 2.7 log cells/ml. The HC5-sensitive bacteria did not adapt and the viable cell numbers were the same after a second dilution ($P > 0.05$).

Butyrivibrio fibrisolvens

B. fibrisolvens are low G + C ruminal bacteria (Willems *et al.*, 1996) that are important in fiber digestion (Hungate, 1966). Only some strains of *B. fibrisolvens* digest cellulose, but virtually all strains can degrade hemicellulose, xylans, pectin and starch. 16S rDNA analyses indicated that *B. fibrisolvens* strains were found in 2 distinct groups (Willems *et al.*, 1996), and later work showed that these groups had distinctly different methods of butyrate production (Diez-Gonzalez *et al.*, 1999). One group had butyrate kinase activity, but the other group had a butyryl CoA/acetyl CoA transferase reaction. In this latter case, ATP was derived from acetate kinase rather than butyrate kinase, and butyrate production was dependent on acetate being present in the growth medium. If acetate was not added, glucose was converted to lactate.

Teather and his colleagues noted that many strains of *B. fibrisolvens* produced bacteriocins that could inhibit other butyrivibrios (Kalmokoff *et al.*, 1996). Approximately 50% of the strains tested produced a bacteriocin, bacteriocin production was not a phylogenically conserved trait, and bacteriocin-producing strains were obtained from both 16S rDNA groups (butyrate kinase versus transferase) (Kalmokoff and Teather, 1997). Teather and his colleagues purified two butyrivibriocins. The *B. fibrisolvens* OR79 butyrivibriocin was a lantibiotic, but the other one (from *B. fibrisolvens* AR10) was homologous to acidocin B, a type IIc bacteriocin produced by *Lactobacillus acidophilus* (Kalmokoff *et al.*, 1997; Kalmokoff *et al.*, 1999). Both of these butyrivibriocins had relatively wide spectra of activity and were able to inhibit a variety of Gram-positive ruminal bacteria. The AR10 butyrivibriocin is sensitive to oxygen, and this property limits its potential use as a feed additive.

N-terminal amino acid analyses showed that the *B. fibrisolvens* OR79 butyrivibriocin was a mixture of two peptides (butyrivibriocin OR79A and OR79B) that differed only with respect to 2 amino acids (Kalmokoff *et al.*, 1999). DNA clones and Southern blot analyses indicated that both peptides were encoded by the same open reading frame (*bvi*79A), and the difference appeared to be post-translational modification. Probes prepared from the N-terminal sequences of the butyrivibriocin OR79 hybridized with chromosomal DNA, and the region adjacent to the structural gene encoded putative immunity proteins, an ABC transporter and a post-translational modifying protein.

N-terminal amino acid analyses indicated that the *B. fibrisolvens* AR10 butyrivibriocin was blocked, but Kalmokoff and Teather (1997) determined the amino acid sequence of a cyanogen bromide cleavage fragment. The AR10 butyrivibriocin gene was part of an operon that contained a histidine kinase-like protein (Genbank Accession number AF076529; Kalmokoff *et al.*, 1997). This latter finding indicates that AR10 butyrivibriocin transcription may be controlled by external stimuli via a two-component regulatory system.

PCR amplification and DNA sequence analyses indicated that 24 out of 39 *B. fibrisolvens* strains had homologues of the butyrivibriocin OR79 structural gene (*bvi*A), and these homologues were even found in non-producing and bacteriocin-sensitive strains (Whitford *et al.*, 2001b). The bacteriocin-producing strains that carried the *bvi*A gene could be organized into 3 different groups (A, B and C), and at least one variant (C) was found in another species (*Butyrivibrio crossotus*) (Whitford *et al.*, 2001b).

Recent work indicated that a strain identified as *B. fibrisolvens* JL5 produced a bacteriocin that inhibited *B. fibrisolvens* AR10, and 16S rDNA analysis indicated that it was distinct from both *B. fibrisolvens* AR10 and OR79 (Rychlik and Russell, 2002). The JL5 bacteriocin catalyzed potassium efflux from *B. fibriosolvens* 49 and caused a decrease in ATP and electrical potential across the cell membrane. The JL5 bacteriocin was degraded by Pronase E, but the rate of this degradation was very slow.

Ruminococcus albus

The early ruminal isolations yielded two cocci with cellulase activity, and they were differentiated by pigment production. The colorless coccus was designated as *Ruminococcus albus* and the yellow one was *Ruminococcus flavefaciens* (Hungate, 1950). Both species produce acetate, but most strains of *R. flavefaciens* produce large amounts of succinate. Ruminococci are predominant cellulolytics that have been readily isolated from domestic and wild ruminants throughout the world, but in vitro experiments indicated that *R. albus* and *R. flavefaciens* could not be co-cultured on cellobiose (Odenyo *et al.*, 1994). *R. flavefaciens* grew faster on cellobiose than *R. albus*, but *R. albus* was the dominant species in co-culture. *R. albus* 8 produced a heat stable protein factor that caused zones of inhibition in *R. flavefaciens* FD1 lawns (Odenyo *et al.*, 1994), and subsequent work indicated that other *R. albus* strains produced bacteriocin-like compounds that could inhibit *R. flavefaciens* strains and *B. fibrisolvens* (Chan and Dehority, 1999).

Lactobacilli

Lactobacilli can be easily isolated from cattle fed grain, but Hungate (1966) concluded that their numbers would not increase until the ruminal pH was already low and *S. bovis* was inhibited. However, when cattle were adapted gradually in stepwise fashion to rations that had an abundance of cereal grain and soybean meal, there was only a modest decrease in ruminal pH, lactate was never detected and lactobacilli out-numbered *S. bovis* (Wells *et al.*, 1997). Because the ruminal pH was always greater than 6.3, the inverse relationship between *S. bovis* and lactobacilli could not be explained by pH per se, but subsequent work indicated that many of the lactobacilli produced a substance that could inhibit the growth of laboratory *S. bovis* strains (Wells *et al.*, 1997). The most active strain was identified as *Lactobacillus fermentum*, and this species was previously reported to produce a bacteriocin (De Klerk and Smit, 1967).

Enterococcus faecium

Enterococcus faecium is not a predominant ruminal bacterium, but bacteriocin-producing *E. faecium* strains have been isolated from the rumen (Lauková and Czikková, 1998; Morovsky *et al.*, 1998). *E. faecium* CCM4231 and BC25 both inhibited *S. bovis*, but the bacteriocin BC25 appears to have a bacteriostatic rather than bactericidal mode of action. *E. faecium* BC25 also inhibited CCM4231, but PCR amplification of the BC25 bacteriocin gene (*ent*A) suggested that both strains had the same 726 bp *ent*A homologue.

Regulation of Bacteriocin Production in Ruminal Bacteria

Many bacteria do not produce bacteriocins in large amount until late exponential or early stationary phase of growth. When Bárcena *et al.* (1998) grew *Lactobacillus plantarum* in continuous culture, bacteriocin activity was maximal at a dilution rate that corresponded to approximately 10–20% of the maximum growth rate, and results with *S. bovis* HC5 indicated that bacteriocin production was catabolite repressed by glucose (Mantovani and Russell, 2002). If the dilution rate was greater than 0.6 h^{-1}, the specific rate of glucose consumption was high enough to inhibit bacteriocin activity.

Lactic acid bacteria are often grown in very rich medium, and it has often been assumed that amino acid supply favors bacteriocin production (Parente and Ricciardi, 1999; Aasen, *et al.*, 2000). *S. bovis* is a bacterium that can utilize ammonia as a sole source of nitrogen (Wolin *et al.*, 1959). When glucose-limited continuous cultures of *S. bovis* HC5 were provided with increasing amounts of peptides and amino acids, the cell mass increased by as much as 35%, but the specific rate of bacteriocin production increased only 2-fold (Mantovani and Russell, 2002). These latter results indicated that exogenous amino acids were stimulatory but not absolutely essential.

Work with lactic acid bacteria indicates that pH can affect the production as well as the activity of bacteriocins, but these effects have not always been differentiated (Yang and Ray, 1994; Biswas *et al.*, 1991). Biswas *et al.* (1991) noted that the pediocin activity of *Pediococcus acidlactici* H was greater if pH was not controlled, and continuous culture experiments with *S. bovis* HC5 indicated that bacteriocin production was maximal at pH 5.5 (Mantovani and Russell, 2002). The activity of nisin is greatly enhanced at pH values less than 6.0, and this result has been explained by an

increase in stability and solubility (Liu and Hansen, 1990). In some cases, the pH effect has been related to changes in the activity of enzymes involved in post-translational modification or to the pH-dependent release of bacteriocins from the cell surface (Biswas *et al.*, 1991; De Vuyst *et al.*, 1996). Further work is needed to define more precisely the effect of pH on activity and stability of ruminal bacteriocins, but preliminary results with *S. bovis* HC5 indicate that low pH has a very positive effect on potassium depletion (Houlihan *et al.*, unpublished results).

The bacteriocins of *S. bovis* HC5 and *S. gallolyticus* LRC0255 are cell associated, but this activity can be released by non-ionic detergents (e.g. Tween 80) or by low pH (e.g. 2.0). However, *B. fibrisolvens* JL5 released its bacteriocin into the cell-free supernatant even if detergents were not added (Rychlik and Russell, 2002). Some butyrivibrios only seemed to produce bacteriocins if they were grown on solid surfaces, but the nature of this regulation has not been precisely defined (Kalmokoff *et al.*, 1996).

Some bacteria use two component regulatory systems to sense small molecules that accumulate extracellularly as the cell density increases (e.g. quorum sensing). Quorum sensing has not yet been demonstrated in ruminal bacteria, but DNA sequencing indicates that *B. fibrisolvens* OR79 has a histidine kinase and response regulator genes adjacent to the structural gene for the butyrivibriocin (Whitford *et al.*, 2001b).

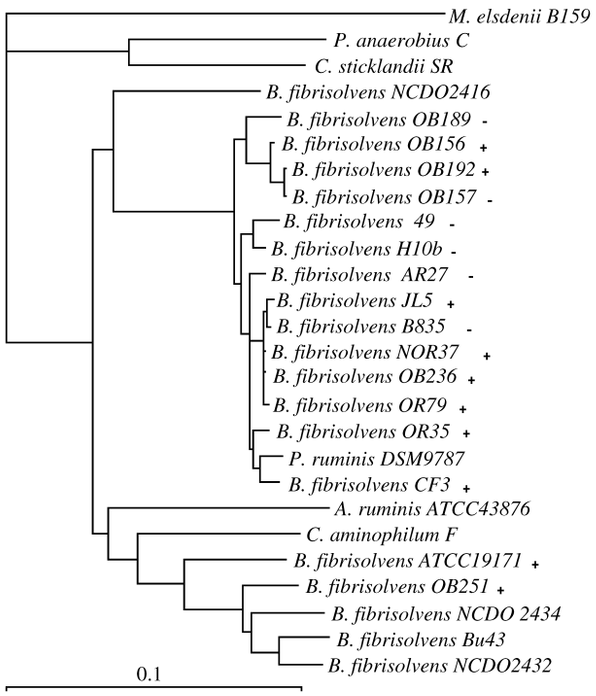

Figure 3. A schematic showing the phylogenic relationships of ruminal *Butyrivibrio* strains based on 16S rDNA. A plus (+) indicates that the strain is known to produce a bacteriocin and a minus (−) indicates that the strain is thought not to produce a bacteriocin.

Phylogeny of Bacteriocin Producing Ruminal Bacteria

The bacteriocin production of ruminal bacteria seems to be confined to Gram-positive bacteria and does not seem to be a phylogenetically conserved trait (Figure 3). When bacteriocin-producing *S. bovis* strains were typed using PCR and repetitive DNA (BOX) sequences, the bacteriocin-producing strains could be organized into 16 groups, and the similarity indexes were as low as 40% (Mantovani *et al.*, 2001). 16S rDNA sequencing indicated that butyrivibriocin production was not confined to a particular group of butyrivibrios, and even highly related species differed in their ability to produce bacteriocins (Figure 3). Recent work, however, indicates that even non-bacteriocin producing strains can carry homologue genes (Morovsky *et al.*, 2001; Whitford *et al.*, 2001a).

Bacteriocin Specificity

Some bacteriocins have a relatively broad specificity (e.g. nisin) and are able to inhibit a variety of bacteria. The bacteriocin-like activity of *S. bovis* HC5 (Mantovani *et al.*, 2001) and *B. fibrisolvens* OR79 inhibited most Gram-positive ruminal bacteria, but butyrivibriocin AR10 was not so broad in its spectrum (Kalmokoff and Teather, 1997). *B. fibrisolvens* JL5 did not inhibit *S. bovis* JB1 or many other *B. fibrisolvens* strains, and *Clostridum aminophilum* was relatively resistant (Rychlik and Russell, 2002).

Bacteriocin specificity may be mediated by cell surface receptors, but the nature of this specificity is not entirely clear. When Zajdel *et al.* (1985) treated *Lactococcus cremoris* 1P5 with trypsin, the cells became 10-fold less sensitive to the lactostrepcin 5 of *L. cremoris* 202. Nisin was not thought to need a receptor, but recent experiments indicate that it binds to lipid II, and nisin activity in membrane vesicles was enhanced when the amount of lipid II was increased (Breukink *et al.*, 1999). Receptors in ruminal bacteria have yet to be demonstrated.

Effect of Bacteriocins on Ruminal Ecology

The effect of bacteriocins on ruminal ecology has not been clearly defined. Because bacteriocin-producing and bacteriocin-sensitive strains can be readily isolated from the rumen, the ability of a bacterium to produce a bacteriocin does not confer an absolute growth advantage (Mantovani *et al.*, 2001). Some strains secrete their bacteriocins into the cell-free supernatant, but bacteriocins are more apt to be cell-associated. Because most ruminal bacteria are attached to feed particles, cell associated bacteriocins could be a critical factor in colonization.

Continuous culture studies (Shi *et al.*, 1997) and in vivo enumerations based on 16S rDNA probes (Weimer *et al.*, 1999) indicated that *R. albus* outnumbered *R. flavefaciens* (a bacteriocin-sensitive species) even though some strains of *R. flavefaciens* grew faster on cellulose in pure culture than *R. albus.*

Chan and Dehority (1999) noted that inhibitory activity of *R. albus* strains was decreased or completely destroyed by the proteolytic activity of *B. fibrisolvens* H15c. However, these studies were based on culture filtrates, and the impact of *B. fibrisolvens* in a tri-culture has not been assessed.

Acute ruminal acidosis is often caused by an overgrowth of *S. bovis,* but *S. bovis* is often replaced by lactobacilli once the ruminal pH is low. Hungate (1966) explained the inverse relationship between *S. bovis* and lactobacilli by differences in pH sensitivity, but Wells *et al.* (1997) showed bacteriocin-producing lactobacilli replaced *S. bovis* even if the ruminal pH was greater than 5.6. Because *S. bovis* and the lactobacilli both grew rapidly at pH values greater than 5.6, pH sensitivity alone could not explain the shift in bacterial ecology. More recent work indicates that *S. bovis* strains can also produce bacteriocins (Whitford *et al.*, 2001a; Mantovani *et al.*, 2001), but the impact of *S. bovis* on lactobacilli has not been addressed.

Obligate amino acid fermenting bacteria appear to play a dominant role in wasteful ruminal amino acid deamination (Rychlik and Russell, 2000), but most probable numbers indicate that these bacteria only represented a very small proportion of the total population (Yang and Russell, 1993). High dilutions of ruminal fluid had glucose-fermenting strains of *B. fibrisolvens* that produced a bacteriocin, and this bacteriocin inhibited the obligate amino acid fermenting bacteria (Rychlik and Russell, 2002). Because mixed ruminal bacteria from cattle fed grain had a much lower specific activity of ammonia production than bacteria from cattle fed hay, and bacteria from cattle fed grain strongly inhibited the ammonia production of obligate amino acid fermenting bacteria, it appears that bacteriocins could play a role in regulating ruminal ammonia production (Rychlik and Russell, 2000).

In the 1970's, Colin Orpin recognized that ruminal microorganisms previously thought to be protozoa were actually fungal zoospores (Orpin and Joblin, 1997). Ruminal fungi are most evident when the diet is primarily fiber, but ruminal fungi are not thought to comprise more than 6% of the total biomass. Recent work by Dehority and Tirabasso (2000) indicated that ruminal bacteria produced a bacteriocin-like substance that inhibited ruminal fungi, and this activity was resistant to proteolytic enzymes.

Applications

Because some ruminal bacteria can produce bacteriocins, Teather and Forster (1998) speculated that these compounds might provide effective alternatives to antibiotics as feed supplements. In order for ruminal bacteriocins to be effective, the peptide would need to: 1) be relatively stable, 2) have a broad spectrum of activity, and 3) remain active (not subject to resistance). Because the rumen is a highly diverse bacterial ecosystem inhabited by many different species (and strains within a species) and bacterial competition is very intense (Whitford *et al.*, 1998; Russell and Rychlik, 2001), inoculation would not necessarily increase the amount of a bacteriocin in the rumen. However, cattle are often fed silages, and silage fermentation is a batch culture system that favors rapidly growing lactic acid bacteria. When Jones *et al.* (1991) inoculated silages, ''*S. bovis* grew faster than any of the commercial species tested and resulted in the most homolactic fermentation.'' Given the observation that some *S. bovis* produce very potent bacteriocins, silage fermentation could be a vehicle for delivering bacteriocins to the rumen (Kalmokoff *et al.*, 1996).

Conclusions

Some bacteria produce small peptides (bacteriocins) that inhibit Gram-positive bacteria. In vitro experiments indicated that the bacteriocin, nisin, could decrease methane and ammonia production in vitro. However, some ruminal bacteria became highly nisin-resistant and nisin can be degraded by mixed ruminal bacteria. A variety of ruminal bacteria produce bacteriocins, and bacteriocin production has, in some cases, been correlated with changes in ruminal ecology. Some ruminal bacteriocins are as potent as nisin in vitro, and resistance can be circumvented. Based on these results, ruminal bacteriocins may provide an alternative to antibiotics used in cattle rations.

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