The Role of Bacteriocins in Rumen Microbial Ecology

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ABSTRACT

In recent years there has been an increased interest in bacteriocins, primarily among the lactic acid bacteria, as a result of their applications in food preservation. There has been very little research on the occurrence of bacteriocin production among anaerobic bacteria. Recently, we reported a high incidence of bacteriocin-like inhibitory activity in isolates of the rumen anaerobe *Butyrivibrio fibrisolvens*: half of the tested butyrivibrio isolates produced bacteriocin-like inhibitory activity, with activities against a wide range of rumen and pathogenic food isolates. We have examined the structure and expression of two of these rumen bacteriocins, butyrivibriocin AR10 and butyrivibriocin OR79A, which represent two structurally different families of bacteriocins. We are also examining the incidence, activities, and structures of bacteriocin-like activities among other groups of rumen bacteria. Our present knowledge of the incidence, characteristics, and range of activity of bacteriocins among the rumen bacteria suggests that bacteriocin production and resistance may play an important role in inter- and intraspecific competition in the densely and diversely populated rumen environment. They may also serve as agents for the directed manipulation of rumen microbial populations.

Introduction

Bacteriocins are ribosomally encoded peptide antibiotics. Both Gram-negative and Gram-positive bacteria produce them. The Gram-negative bacteriocins were the first studied in any detail. Best known are the colicins, which are produced by, and attack, strains of *E. coli* (4). These are large, complex proteins, 29-90 kDa, with characteristic structural domains involved in cell attachment, translocation, and bactericidal activity. They bind to specific receptors on the outer membrane of the target cell. As a consequence, their range of activity tends to be very narrow.

The bacteriocins produced by Gram-positive bacteria, on the other hand, are in most cases small peptides, 3-6 kDa in size (21), although there are exceptions (14). They fall within two broad classes: the lantibiotics (13) and the non-lantibiotic bacteriocins (21). All undergo a post-translational modification involving cleavage of a leader peptide (13, 21). In lantibiotics, additional post-translational modifications to specific amino acids also occur, giving rise to a variety of unusual amino acid residues that include unsaturated amino acids, thioether-linked diamino acids, and a number of other modified amino acid residues (26, 13, 17). Both classes of peptide antibiotics demonstrate great heterogeneity in terms of amino acid sequence and the susceptibility of target species.

Most of the Gram-positive bacteriocins that have been investigated are membrane active compounds that increase the permeability of the cytoplasmic membrane (13). They often

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show a much broader spectrum of bactericidal activity than the colicins; this may be related to their mode of action, the lack of a requirement for a specific cell surface receptor, and/or the absence of an outer membrane to restrict their access to the cytoplasmic membrane. Many, however, are much more restricted in their range of activity.

There is currently much interest in the application of bacteriocins in both food preservation and the inhibition of pathogenic bacteria (20, 29, 5). Most of the bacteriocins that have been studied extensively were originally isolated from organisms involved in food fermentation. Bacteriocin production and resistance is considered an important property in strains used as commercial inoculants to eliminate or reduce growth of undesirable or pathogenic organisms. When environmental isolates have been examined, between 1% and 10% of the isolates have been identified as producers of bacteriocin-like inhibitory substances (BLIS).

The microbial population of the rumen includes a very dense bacterial population of 10^{10} to 10^{11} bacteria/ml. The extent of bacterial diversity in the rumen is still not completely defined, but estimates of diversity have increased dramatically over the last decades. Groups of strains that were formerly classed on a phenotypic basis into a single species, such as *Butyrivibrio fibrisolvens*, are now known to be quite diverse phylogenetically (9, 28, 8).

A consequence of this high level of diversity and high population density, of course, is a high level of opportunity for interaction between cells, and, presumably, intense competition between the many organisms that have a similar phenotype but different genotype. The fact that the rumen is a contained environment, with a significant level of mixing, means that there is opportunity for both direct and indirect interactions between all parts of the microbial population.

Under these circumstances, the production of antimicrobial compounds, such as bacteriocins, and the possession of resistance mechanisms against antimicrobial compounds produced by other organisms, could be both a very effective way of securing a niche in the environment, and essential for survival.

Bacteriocin production by rumen bacteria

Examples of BLIS have been noted in one strain of *Streptococcus bovis* (12), a number of strains of *Enterococcus* and *Staphylococcus* isolated from calves (18), and from a single isolate of the rumen anaerobe *Ruminococcus albus* (22). None of these BLIS were confirmed to be true bacteriocins.

More recently, we tested 49 isolates of *B. fibrisolvens* for BLIS production; 25 of the 49 strains produced BLIS (15). The Butyrivibrio BLIS showed a wide range of variation in activity, from inhibitors affecting only a few closely related strains of *Butyrivibrio* to inhibitors that affected essentially all tested Gram-positive bacterial genera. Preliminary surveys of other groups of Gram-positive rumen bacteria indicate that they too may represent a source of new bacteriocins that could find application in the manipulation of rumen fermentation. Of 30 strains of *S. bovis* examined, about 25% produced BLIS, while among strains of *Selenomonas ruminantium* the frequency of BLIS production was about 5% (Whitford and Teather, unpublished). In general, the incidence of bacteriocin production among the groups of rumen bacteria examined to date is higher than that observed in other environments.

Characterization of rumen bacteriocins

To investigate the nature of the BLIS produced by rumen butyrivibrios, we have cloned, sequenced, and analyzed the expression of the genes encoding two of the inhibitors.

Butyrivibriocin AR10

One of the characterized BLIS, from *B. fibrisolvens* AR10, appeared on the basis of N-terminus amino acid sequence to be a type IIc bacteriocin. The isolated BLIS demonstrated the same antimicrobial spectrum as the producing strain, with a wide spectrum of activity among *Butyrivibrio* isolates but a relatively limited spectrum of activity against other genera (15). A single sequence, encoding a peptide of 80 amino acids (BviD), was identified in the genome of *B. fibrisolven*s AR10 using a probe based on the amino acid sequence of a CNBr fragment of the isolated BLIS. The predicted amino acid sequence of BviD was identical with the determined internal amino acid sequence in seventeen of twenty consecutive positions. Although three differences were found between the determined N-terminal sequence and the cloned gene (two blank cycles, one amino acid discrepancy), these fall within the margins of error normally associated with amino acid sequence determinations. The single amino acid discrepancy, found in position 18 of the determined internal peptide sequence, was an alanine for serine substitution. A similar substitution has been reported in the lantibiotic lactocin S, where three encoded serines are converted to D-alanine during maturation (26). There was also a significant correlation between the determined amino acid composition (15), and that predicted by *bviD*. The predicted peptide sequence demonstrated homology with acidocin B, an unusual bacteriocin produced by *Lactobacillus acidophilus* (19). Transcriptional analysis indicated that expression of *bviD* occurred at high levels in cells grown on L10 plates or in high glucose liquid L10 medium, but not in cells grown using standard L-10 liquid medium, exactly mirroring the conditions for bacteriocin production in cultures (15).

Eight ORFs were identified in the cloned region encompassing the bacteriocin gene (Fig. 1). Both Northern blotting and primer extension analysis indicated that the region encompassing ORFs 3-7 forms an independent transcriptional unit (*bviABCDE*). This operon has a number of features.

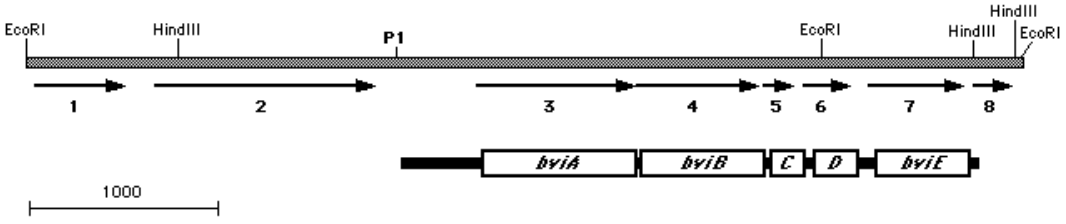

Fig. 1. Butyrivibriocin AR10 operon. Numbers 1 through 8 are open reading frames identified by sequence analysis. Transcription of the operon begins at P1 and proceeds through *bviA* to the end of *bviE*. The bar corresponds to 1000 bp.

Four genes were co-transcribed with the bacteriocin (*bviD*). There was a marked difference in the abundance of transcripts encompassing portions of the operon. Transcripts encompassing the bacteriocin gene (*bviD*) and *bviE* only were present at much higher levels than transcripts encompassing the entire operon. The observed differences in the relative stability of various portions of the transcript suggest that translation within the operon may be regulated, in part, at the level of mRNA stability (1).

Transcription was initiated 500 bp upstream of the start of *bviA* (P1: Fig. 1). The region between P1 and the start of *bviA* contained a number of structural features, including a significant number of direct repeats and two inverted repeats. Significant numbers of both direct and inverted repeats have also been found within the promoter regions of the two ORFs found on a cryptic plasmid isolated from *B. fibrisolvens* (10). Repetitive elements have been noted preceding the promoter regions in the plantaricin A (6), carnobacteriocin B2 (24), and sakacin A (2) bacteriocin operons, and palindromic sequences precede the promoters in several of the epidermin operons and the promoter for the gene encoding sakacin P (27). The palindromic sequence in the epidermin operon appears to represent the recognition site for EpiQ, a transcriptional activator of these operons (23). In the case of the butyrivibriocin AR10 operon, the structural features follow the apparent transcription initiation site. These findings suggest that regulation of expression among butyrivibrios may be somewhat different than that previously reported in other bacteriocin systems (6). If this region is a binding region for transcriptional regulators, its location downstream of the promoter initiation site suggests that transcription of the bacteriocin operon may be regulated via a repressor rather than through transcriptional enhancers.

Minor transcripts that initiated at positions further upstream of this site were also present within the total RNA pool. There were two additional ORFs (ORFs 1 and 2, Fig. 1), with significant homologies to previously reported two-component regulatory systems, directly upstream of the *bviABCDE* promoter. Two-component regulatory systems are involved in the regulation of bacteriocin synthesis in several isolates (3, 16, 11, 21, 24). While ORFs 1 and 2 appear to form a portion of a separate operon, primer extension analysis indicated a low level of transcription from this operon into the bacteriocin operon. The direct linkage with the bacteriocin operon, the low levels of transcription which initiate upstream of the bacteriocin promoter initiation site, and the homologies with other bacteriocin two component regulatory systems, suggest that ORFs 1 and 2 may be involved in the regulation of butyrivibriocin AR10 production.

The function of the four genes cotranscribed with the bacteriocin have not been determined, but can be surmised. Bacteriocin producers are immune to their own bacteriocin, which results from the presence of a specific immune protein. Each bacteriocin requires a separate immune protein, and there appears to be little sequence homology among those characterized to date (21). In all known cases, the gene encoding the immunity protein of type II bacteriocins is co-transcribed with, and produced in a one to one ratio with, the bacteriocin (21). Both the direct linkage and the relative abundance of the transcript encompassing *bviDE* suggest that BviE functions as the immunity protein. The hydrophobic character and prediction of membrane spanning domains, which indicate an envelope location, are both consistent with a function as an immunity protein.

BviA, BviB, and BviC may form an ABC transport system. BviA falls within the superfamily of ABC transporters (7). ABC transporters consisting of three components (a peripheral protein containing the ATP-binding cassette and two integral membrane proteins) are found in a number of bacterial ABC importers (7), and a similar organization is found with the proteins involved in immunity to bacitracin (2) and certain lantibiotics

(25). Coincidentally, these ATP-binding proteins demonstrated the highest homology matches with BviA. Both BviB and BviC are hydrophobic and contain predicted membrane spanning domains (6 and 2 respectively), and therefore would be expected to have an integral membrane location. Furthermore, both are directly linked with *bviA*, and transcripts containing these three genes are present at relatively low levels compared to *bviDE*. We suggest that these three components form an ABC transport system.

Butyrivibriocin OR79

The rumen anaerobe *Butyrivibrio fibrisolvens* OR79 produces a bacteriocin-like activity demonstrating a wide range of activity against butyrivibrios, other rumen bacteria, and pathogenic food bacteria. An inhibitor with the same antimicrobial spectrum as the producing strain was isolated from spent culture fluid using a combination of ammonium sulfate and acidic precipitation, reverse phase chromatography, and high resolution gel filtration.

N-terminal analysis of the isolated inhibitor yielded a 15 amino acid sequence (G-N/Q-G/P-V-I-L-X-I-X-H-E-X-S-M-N). Two different amino acid residues were detected in the second and third positions of the N-terminus, indicating the presence of two distinct peptides. A gene with significant homology to one combination of the determined N-terminal sequence was cloned, and expression of the gene confirmed through Northern blotting.

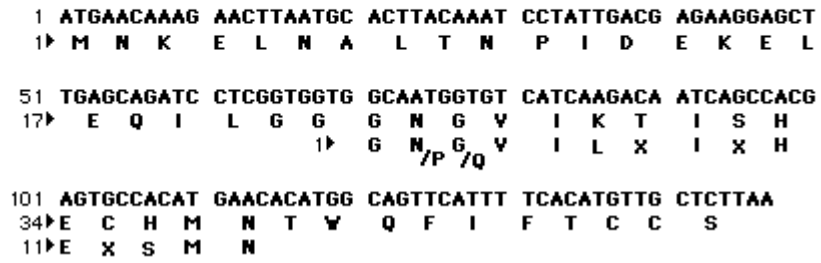

Fig. 2. Sequence of the butyrivibriocin OR79A gene, *fibA*. The N-terminal amino acid sequence of the isolated peptides is shown underneath the predicted amino acid sequence.

The gene (*fibA*) encoded a pre-peptide of 48 amino acids, and a mature peptide, butyrivibriocin OR79A, of 25 amino acids (Fig. 2). Butyrivibriocin OR79A showed no significant homology with butyrivibriocin AR10. Significant sequence homology was found between this peptide and previously reported lantibiotics containing the double glycine leader peptidase processing site. Immediately downstream of *fibA* was a second partial open reading frame encoding a protein with significant homology to proteins that are believed to be involved in the synthesis of lanthionine residues. These findings indicate that the isolated inhibitory peptides represent new lantibiotics. Results from both total and N-terminal amino acid sequencing indicate that the second peptide was identical to butyrivibriocin OR79A except for amino acid substitutions in positions 2 and 3 of the mature lantibiotic. At this time it is not known whether the second lantibiotic, butyrivibriocin OR79B, is the product of a second gene or results from the direct modification of butyrivibriocin OR79A.

Implications for rumen microbial ecology

Bacteriocins representing both major groups of peptide antibiotics have now been characterized from *B. fibrisolvens* isolates. Given that these bacteriocins represent only two from a large number of isolates which demonstrated bacteriocin-like activities, the bacteriocins produced by isolates of *B. fibrisolvens* may be as diverse as those found within the lactic acid bacteria, and may represent a good source for new bacteriocins.

The high incidence and wide range of activities noted among the rumen bacteriocins characterized to date suggests that bacteriocin production and resistance may play an important role in inter- and intraspecific competition in the densely and diversely populated rumen environment.

Rumen-derived bacteriocins may find direct practical application in areas related to ruminant production, in the same way that ionophore antibiotics are used today. In this application the bacteriocins offer the advantages of a wider range in specificity, a minimal residue problem, and the possibility of production either *in situ* or in fermented feeds.

For those interested in the colonization of the rumen with engineered or exogenous bacteria, the high incidence of bacteriocin production among rumen bacteria presents both problems and opportunities. Any strain that is going to be introduced successfully will almost certainly need to be resistant to endogenous bacteriocins. Bacteriocins may serve as selective agents for establishing a new strain in the rumen, and inoculants that themselves produce bacteriocins could be used both to kill, and then to prevent regrowth (or establishment), of competing bacteria from either endogenous or exogenous sources.

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