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Mutants of *Prevotella ruminicola* Defective in Peptidase Activity: Impact on Ammonia Production

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An enzyme from a predominant ruminal bacterium that degrades peptides has a significant impact on ammonia production, and its manipulation could increase efficiency of nitrogen retention in forage-fed animals.

Summary

*Two **P. ruminicola** mutants defective in peptidase activity were obtained using a method of chemical mutagenesis, and the relevance of this activity in terms of ruminal ammonia production was demonstrated by co-culture experiments. The 25 percent decrease in ammonia production when Gly-Arg-MNAse is absent illustrates the interspecies association regarding degradation of protein sources in the rumen. These mutants will be useful for future studies of this activity, and also demonstrate how molecular biology techniques can be applied in the quest to improve efficiency of beef production.*

Introduction

Considering that up to 25 percent of the feed protein fed to ruminants may be wasted by excessive ammonia production in the rumen, manipulation of ruminal protein digestion could have a positive effect on nitrogen retention efficiency in ruminants, as well as decrease nitrogen losses via animal waste. Much effort has been directed towards identifying and evaluating sources of bypass (escape) protein, as well as identifying which microorganisms are involved with protein digestion and ammonia production. Little information is available about the characteristics and contribution of

protein-degrading enzymes present in ruminal contents. Such information is critical to the development of new, improved strategies to control the rate of forage protein digestion. Molecular biology techniques provide the tools to achieve such a goal. For instance, the mutational analysis of an enzyme can effectively show the relevance of that enzyme related to nutrient utilization, growth and development of the microorganism. With specific emphasis upon ruminal ammonia production, the dipeptidyl peptidase activity of *P. ruminicola* described in the adjoining paper is considered to be the predominant peptidase activity in the rumen. This enzyme is thought to have a major role in controlling protein digestion to small peptides and amino acids that are subsequently broken down to ammonia and VFA by other ruminal bacteria. By removing such activity from this predominant ruminal bacterium the rate and(or) extent of ammonia production from various protein sources should be reduced. Following is a description of how such a hypothesis was tested, and the conclusions we obtained from these experiments.

Procedure

Mutagenesis of ***P. ruminicola***

P. ruminicola strain $B_1$4 was cultured overnight in rich medium, and then diluted 1:20 into defined medium. The mutagen ethylmethylsulfonate (EMS) was added to a final concentration of 0.05% (v/v) and the broths were incubated at 37°C for 5, 10, 15, 30, 45 and 60 minutes. Treated and control cultures (no EMS added) were centrifuged and the cell pellets were washed twice with sterile defined medium to remove residual mutagen. The mutagenized cells were then resuspended in defined medium, and aliquots were taken for serial dilutions and plate counts to determine the number of viable cells remaining following mutagenesis. The remaining cell suspension was then incubated overnight at 37°C.

After overnight growth, the mutagenized cultures were plated again to quantify the rate of mutagenesis. This was done by spreading serial dilutions of the cultures on plates of rich agar medium with or without 20 μg ml^{-1} rifampicin added.

Selection of mutants

To identify mutants lacking dipeptidyl aminopeptidase-like activity, plates inoculated with 200-300 mutagenized colonies were overlaid with a soft-agar solution containing 2 mM glycyl-argininyl-methylnapthylamide (Gly-Arg-MNA). The MNA group is attached to the Gly-Arg dipeptide via a peptide bond. Therefore, only those enzymes capable of cleaving a peptide bond, and allowing Gly-Arg to bind to it, will degrade this substrate. As such, Gly-Arg-MNA is a very specific substrate for the peptidase we are studying. The agar overlay was allowed to solidify and then the plates were left protected from light in the incubator at 37°C. After 30 minutes, the plates were positioned under a long wave ultraviolet lamp. Mutant colonies were not expected to produce the fluorescent "halo" indicative of cleavage and release of free-MNA from the dipeptide conjugate. Putative mutants isolated by this method were quantitatively assayed for the loss of Gly-Arg-MNAse activity using a standardized method (see adjoining paper) to confirm the phenotype.

Characterization of mutants

The impact of the mutation(s) on growth of wild type (WT, i.e. still possessing Gly-Arg-MNAse) and mutant strains (GM4 and GM6, i.e. deficient in Gly-Arg MNAse activity) was measured by growing them in defined medium prepared to contain different nitrogen sources: either 10 mM ammonia, peptides (1.25% w/v Trypticase), or ammonia plus peptides (10 mM ammonia and 1.25% Trypticase), all in the presence of 0.4% glucose. Growth was assessed by optical density (OD_{600})using a spectrophotometer.

Co-cultures of ***P. ruminicola*** *wild type and Gly-Arg-MNAse mutants with ammonia-producing organisms*

To demonstrate the relevance of this peptidase activity in the ruminal environment, co-cultures of the wild type and mutants, along with two ruminal isolates that are known to possess high rates of ammonia production from amino acids or small peptides, were established in a medium containing clarified rumen fluid (5%) and large quantities of peptides from two sources: Trypticase and Gelatin hydrolyzate. Before inoculation with strain C (*Peptostreptococcus anaerobius*) or strain F (*Clostridium aminophilum*), tubes were inoculated with anaerobically harvested *P. ruminicola* cells (wild type and mutants), to a final concentration of 400 mg protein/liter. Monocultures of all organisms were established under the same conditions provided for the co-cultures. Results presented are the average of two independent experiments, with duplicate incubations of each treatment per experiment. A second set of experiments was also conducted with the same number of experimental observations but with the inclusion of 0.1% glucose in the medium. Co-cultures were incubated at 37°C. Samples (1.2 ml) were collected anaerobically at time 0, 12, 24, 48, and 72 hours for ammonia analysis. Ammonia was determined colorimetrically using the phenol-hypochlorite method on an AutoAnalyzer II.

Results

Mutagenesis of ***P. ruminicola*** *and characterization of mutants*

The broth containing cells incubated for 45 minutes in the presence of the mutagenic compound EMS was chosen to be screened for the mutants defective in the peptidase activity, based on the adequate survival (50%), and highest frequency of mutation, as measured by the acquired resistance to the antibiotic rifampicin (data not shown). Approximately 6,000 colonies were screened

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Table 1. Gly-Arg-MNAse activity of *P. ruminicola* B_14 wild type and mutants, expressed in nmol/min/mg protein. Values in parentheses represent generation time expressed in minutes.[1]

	Nitrogen Source		
	10 mM ammonia	1.25% peptides	Ammonia plus peptides
Wild type	6.7(65)	9.3(67)	7.0 (68)
Mutant GM4	0.7(68)	0.7(69)	0.6 (67)
Mutant GM6	0.7(67)	0.8(69)	0.7 (66)

[1] Results are average of four observations.

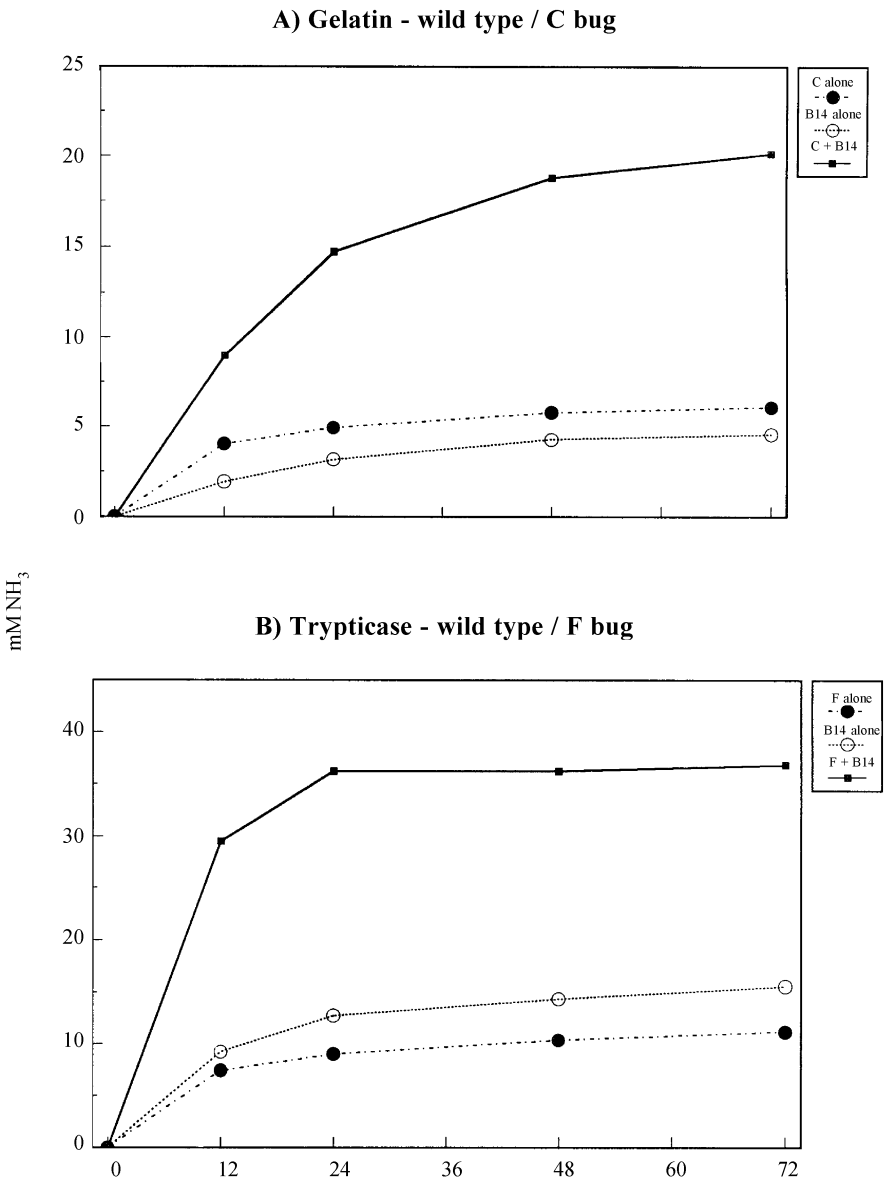

Figure 1. Ammonia production by *Prevotella ruminicola* wild type growing on gelatin hydrolyzate or Trypticase, and by strains C (on gelatin) and F (on Trypticase), growing as monocultures and co-cultures. Results are average of two experiments, with two observations per time incubation time.

and two mutants were confirmed to be defective in peptidase activity, and are identified as GM4 and GM6.

Characterization of mutants

Mutants possessed approximately 10 percent of the peptidase activity measurable from the wild type (Table 1). Little activity was found in the cell-free supernatants, and values were similar for WT, GM4 and GM6. The total activity of the mutants, when measured using cell fragments, was also decreased by 10 times the activity of the wild type (data not shown). These results confirm the mutant strains truly are deficient in Gly-Arg-MNAse activity, rather than a change in the location of enzyme activity. Growth rates of both mutants in media containing either 10 mM ammonia, 1.25% Trypticase peptides, and ammonia plus peptides were similar to those obtained with the wild type under the same conditions (Table 1). In addition, total cell yield of mutants did not differ greatly from that for the wild type, reflected in similar final OD_{600} values in all cultures. Such findings are promising, because they suggest that if Gly-Arg-MNAse could be inhibited, ammonia production might be changed, but the growth and useful activities of *P. ruminicola* in the rumen might still be retained.

Ammonia production by co-cultures

Ammonia production by co-cultures of the wild type *P. ruminicola* strain with either ammonia-producing strain C or strain F showed a more than additive effect when compared to the monocultures (Figure 1), supporting the role of *P. ruminicola* in providing the substrate for strains C and F to produce ammonia. Co-cultures of strains C and F with mutants defective in Gly-Arg-MNAse activity showed a significant (approximately 25%) decrease in ammonia production when compared with incubations in the presence of the wild type (Figure 2). Incubations in the presence of glucose showed a slightly higher rate and extent of ammonia production,

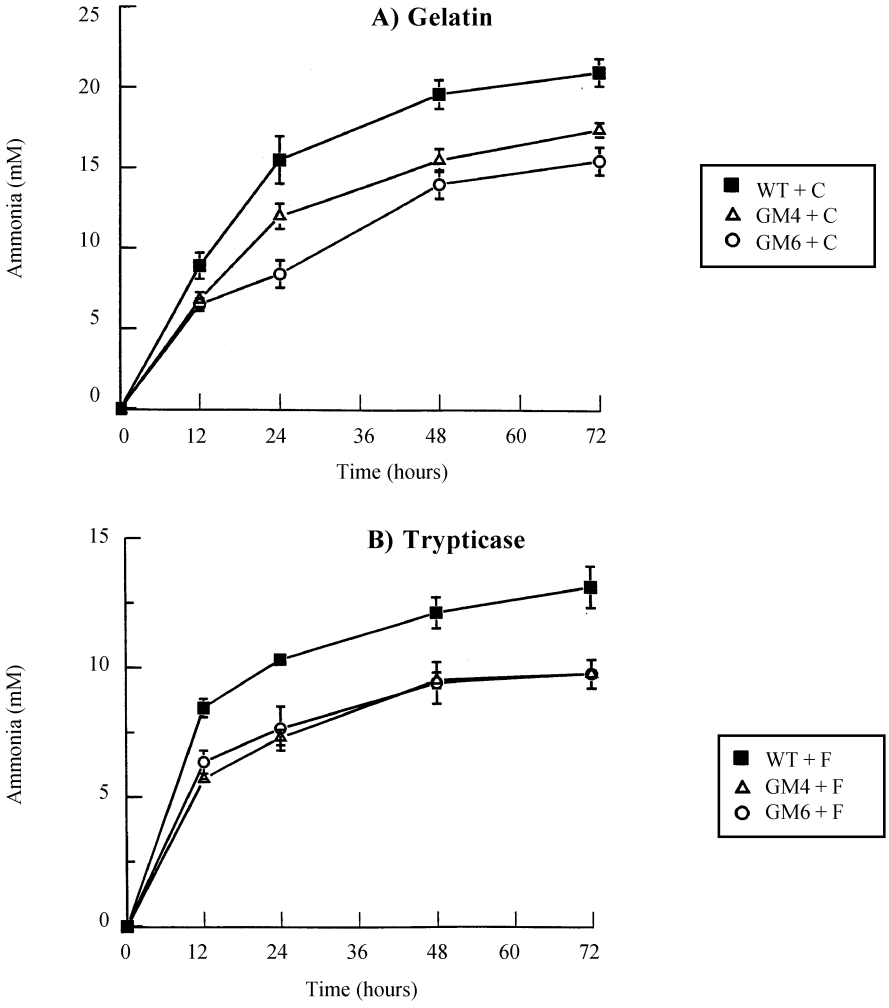

Figure 2. Ammonia production of co-cultures of *P. ruminicola* wild type and mutants (GM4, GM6) with either strain C on gelatin hydrolyzate, or strain F on Trypticase. Results are average of two experiments, with two observations per incubation time.

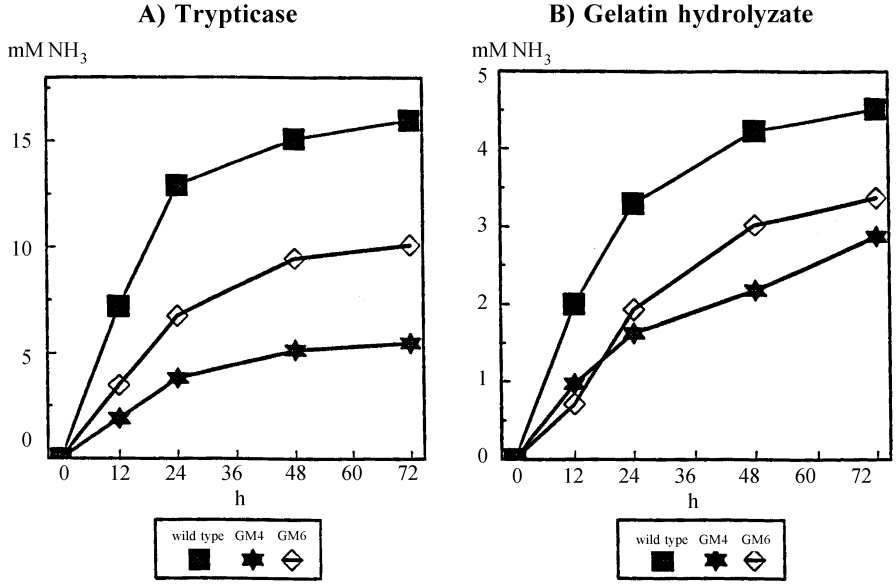

Figure 3. Ammonia production by *P. ruminicola* wild type and mutants (GM4, GM6) on gelatin hydrolyzate or Trypticase. Results are average of two experiments, with two observations per incubation time.

but the degree of contribution of the mutants to the patterns of ammonia production by the co-cultures was similar to that observed in the absence of glucose.

Interestingly, *P. ruminicola* mutants defective in Gly-Arg-MNAse showed decreased rate and extent of ammonia production, a decrease ranging from 35% (gelatin hydrolyzate) to 60% (Trypticase), when compared to the wild type (Figure 3). Further research is needed to demonstrate whether the mutation(s) imposed also affected genetic material coding for deaminase(s), or if the reduced ammonia production is a function of the reduced supply of substrate for deamination.

Conclusions

The generation of two mutants of *P. ruminicola* $B_1 4$ defective in peptidase activity was successfully achieved using a chemical mutagenesis protocol. The ecological relevance of this is demonstrated by its impact on ammonia production by the ammonia-producing ruminal bacteria *Peptostreptococcus anaerobius* and *Clostridium aminophilum*, when co-cultured with mutants of *P. ruminicola*. Co-cultures with the mutants showed a 25 percent decrease in ammonia production when compared to the wild type. The development of inhibitors specific for Gly-Arg-MNAse activity should positively affect the nitrogen balance of beef animals: either by increasing the escape value of forage protein, or reducing the losses of ammonia in animal waste.

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