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Biochemical and Mutational Analysis of a Gingipain-Like Peptidase Activity from *Prevotella ruminicola* $B_1$4 and Its Role in Ammonia Production by Ruminal Bacteria†

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A chemical mutagenesis protocol was used with the ruminal bacterium *Prevotella ruminicola* strain $B_1$4 to generate mutant strains defective in peptidase activity. Compared with the wild-type parent strain, the isolated mutants possessed 1/10 of the enzyme activity responsible for cleavage of glycine-arginine-4-methoxy-β-naphthylamide (Gly-Arg-MNA). A concomitant loss in activity against arginine-arginine-4-methoxy-β-naphthylamide (Arg-Arg-MNA) was also observed. Both activities were similarly affected by various proteinase inhibitors, suggesting that the same enzyme is responsible for the Arg-Arg-MNA peptidase and Gly-Arg-MNA peptidase activities. Growth rates of wild-type and mutant strains grown in batch culture with various nitrogen sources did not differ. However, a role for the Gly-Arg-MNA peptidase activity was demonstrated in coculture experiments with gram-positive, ammonia-producing ruminal bacteria. The rate and extent of ammonia production were reduced by approximately 25% in cocultures containing the mutants when compared with that of wild-type-containing cultures. These reductions could not be accounted for simply by the decrease in ammonia production by the mutant strain alone. To our knowledge, this paper reports the first successful use of chemical mutagenesis with ruminal microorganisms.

As the management practices associated with meat and milk production continue to become more intensive, so too does the need to minimize nutrient excretion in animal waste. A particular case in point is the practice of feeding protein-rich forages to ruminant animals; ammonium (NH_4^+) is often produced at a rate which exceeds its assimilation by the ruminal bacteria. This excess is ultimately excreted into the environment and can represent up to 25% of the nitrogen equivalents fed to the animal (17, 21). Ruminal ammonia production rates can be accounted for by the combined activities of proteolytic bacteria such as *Prevotella ruminicola* as well as several species of gram-positive amino acid-fermenting bacteria (2, 22). When grown as cocultures in the presence of a protein source, these bacteria act synergistically: the proteolytic activities of *P. ruminicola* produce the appropriate substrates (dipeptides and amino acids) for deamination by gram-positive ammonia-producing bacteria (2). Therefore, methods which manipulate ruminal protein and peptide degradation by these bacteria might have a major impact on efficiency of nitrogen retention in livestock. However, little mechanistic or quantitative information is available about specific enzymes thought to be relevant in controlling rates of ruminal ammonia production. The major peptidase activity measurable in ruminal contents is a diaminopeptidyl peptidase, assayed with the substrate glycine-arginine-4-methoxy-β-napthylamide (Gly-Arg-MNA). This Gly-Arg-MNA peptidase (Gly-Arg-MNAse) activity has been proposed to possess a prominent role in ruminal protein degradation (25), and bacteria currently classified as *P. ruminicola* are presumed to be the primary source of such enzyme activity (26). In this study, we adapted a chemical mutagenesis protocol used primarily with enteric bacteria to generate mutants of *P. ruminicola* $B_1$4 which were defective in Gly-Arg-MNAse enzyme activity. In addition to performing a preliminary biochemical analysis of this enzyme activity, we were able to assess the role of such enzyme activity in affecting the kinetics of ammonia production by the predominant ruminal bacteria.

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MATERIALS AND METHODS

Bacterial strains and growth conditions. All bacterial strains used in this study were kindly provided by M. A. Cotta, USDA, NCAUR, Peoria, Ill. *P. ruminicola* $B_1$4 was maintained in either a rumen fluid-containing maintenance (EM) medium (1) or a defined medium as described previously (4, 12). The gram-positive ammonia-producing bacteria *Peptostreptococcus anaerobius* and *Clostridium aminophilum* were cultivated in semidefined medium (2) with the exception that clarified ruminal fluid (5%, vol/vol) was also included. Either 1.5% (wt/vol) gelatin hydrolysate (U.S. Biochemicals, Cleveland, Ohio) or 1.5% (wt/vol) Trypticase (BBL, Becton Dickinson, Cockeysville, Md.) was used as a nitrogen source, and the cultures were transferred every other day. All cultures were incubated at 37°C. When necessary, 1.5% (wt/vol) Bacto Agar (Difco Laboratories, Detroit, Mich.) was included.

For peptidase assays, the wild-type and mutant strains of *P. ruminicola* $B_1$4 were grown in 10-ml batch cultures of defined medium containing either 10 mM ammonium chloride, ammonia-free peptides (1.25% [wt/vol] Trypticase, prepared as described in reference 20), ammonium chloride plus peptides (at concentrations of 10 mM and 1.25% [wt/vol], respectively), or 0.25% (wt/vol) gelatin (porcine skin, PCR grade; U.S. Biochemicals). Glucose was added to a final concentration of either 0.1 or 0.4% (wt/vol). Fresh medium was inoculated with 0.2 ml of overnight cultures grown in the same medium. Growth was measured by the increase in optical density at 600 nm (OD_{600}) of cultures (18- by 150-mm tubes) with a spectrophotometer (Spectronic 20D; Milton Roy Co., Rochester, N.Y.).

EMS mutagenesis of *P. ruminicola* $B_1$4. The procedures described by Miller (15) were used but adapted for use under anaerobic conditions. Briefly, *P. ruminicola* $B_1$4 was cultured overnight in EM medium and then diluted 1:20 into seven prewarmed tubes of defined medium. The mutagen ethyl methanesulfonate (EMS; Sigma Chemical Co., St. Louis, Mo.) was added to each of these tubes to give a final concentration of 0.05% (vol/vol), and then the tubes were placed in an incubator, resulting in the exposure of the cells to EMS for 5, 10, 15, 30, 45, and 60 min. The treated and control cells (no EMS added) were then harvested by centrifugation (10,000 × *g*, 10 min, 4°C), washed twice with 5-ml volumes of sterile, anaerobic defined medium to remove residual mutagen, and resuspended in 5 ml of fresh medium. At this point, aliquots of the washed cells

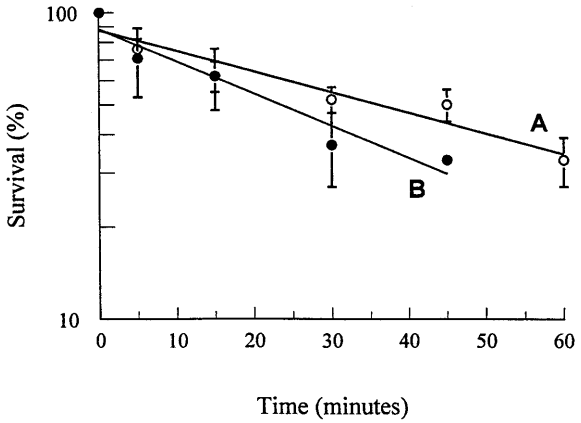

FIG. 1. Plot of the survival of *P. ruminicola* B_14 cells following exposure to EMS for various time periods. Two initial cell densities were used, namely, 6×10^8 CFU ml^{-1} (line A) and 2×10^8 CFU ml^{-1} (line B). For each cell density, the values represent the means (± standard errors of the means) of the initial viable cell count which is still viable after EMS treatment, determined from plate counts made in duplicate from two independent cultures.

were taken for serial dilution with an anaerobic diluent, and 0.1-ml volumes were subsequently plated onto EM-agar medium to determine the number of viable cells remaining following mutagenesis. The remainder of the mutagenized cultures were placed in the incubator and allowed to grow overnight.

After overnight growth, aliquots of the mutagenized cultures were diluted and spread on EM-agar medium with or without 20 μg of rifampin (Sigma) ml^{-1} added to quantify the rate of mutagenesis (15). These plates were incubated for 48 h, and during this time, the remainders of the cell cultures were kept at 4°C.

Selection of Gly-Arg-MNAse-deficient mutants. Aliquots (0.1 ml) of the mutagenized cells were spread on defined agar medium, which contained 5 mM ammonium chloride as the sole nitrogen source. After 48 h of incubation, when colonies reached a diameter ranging from 1 to 3 mm, the plates were overlaid with a sterile, anaerobically prepared solution of agarose (1%, wt/vol), containing 25 mM phosphate buffer (pH 7.5) and 2 mM Gly-Arg-MNA (Sigma). The overlay was allowed to solidify, and the plates were left protected from light in the incubator for 30 min. To screen for the presence of Gly-Arg-MNAse activity, the plates were positioned briefly under a long-wave UV lamp (Blak-Ray UVL-22; Ultra Violet Products Inc., San Gabriel, Calif.). Mutant colonies were not expected to produce a blue fluorescent halo, indicative of cleavage and release of free MNA from the dipeptide conjugate.

Cell fractionation and enzyme assays. The cells were harvested either in the mid-logarithmic phase of growth or after overnight growth, and peptidase assays were performed with whole cells as well as cellular fractions. To prepare such fractions, 50-ml cultures were centrifuged as described previously and the cell pellet was resuspended in 5 ml of anaerobically prepared, 25 mM phosphate buffer (pH 7.5). The cells were then broken by two passages through a French pressure cell (SLM Instruments, Inc., Urbana, Ill.) set at 208,000 kPa, collected in a test tube kept on ice, and purged with a continuous flow of N_2 gas. Following cell disruption, a low-speed centrifugation under anaerobic conditions was used to remove any unbroken cells and large debris. The resulting supernatant was then centrifuged at 350,000 × *g*, at 4°C, for 30 min in an Optima-TLX ultracentrifuge (Beckman Instruments, Palo Alto, Calif.). Following ultracentrifugation, the samples were immediately transferred to an anaerobic chamber (Coy Products, Ann Arbor, Mich.) and the supernatant (cytoplasmic-periplasmic fraction) was separated from the pellet (membrane fraction), the latter of which was resuspended in phosphate buffer. These fractions were transferred to 2-ml screw-cap, cryostorage vials and placed in a liquid N_2 storage tank until analyzed for Gly-Arg-MNAse activity.

Gly-Arg-MNAse activity was assayed as described previously (25), with the exception that the buffer was saturated with N_2 gas, and all enzyme assays were conducted within an anaerobic chamber containing a 95:5 (vol/vol) N_2-H_2 atmosphere. In addition to Gly-Arg-MNAse activity, Arg-Arg-MNAse, Gly-Pro-MNAse, and Lys-Ala-MNAse specific activities were also measured. All substrates were obtained from Sigma, and enzyme specific activity is expressed as the nanomoles of substrate cleaved per minute per milligram of protein. Protein concentration was determined by the method of Lowry et al. (11) with bovine serum albumin as a standard. To test the effect of various inhibitors on enzyme activity, samples (whole cells or cell fractions) were incubated in the presence of inhibitors at 37°C for 10 min prior to the addition of the substrates to individual reaction mixtures; for the concentrations of inhibitors used in these assays, see Table 3. Phenylmethylsulfonyl fluoride (PMSF), the sodium salt of *p*-chloromercuribenzoate (pCMB), iodoacetic acid (IAA), EDTA, and EGTA were all obtained from Sigma. The effect of aerobic conditions on enzyme activity was tested by incubating either whole cells or cell fractions with the substrate outside the anaerobic chamber. The requirements for reduced sulfhydryl groups was assessed by the addition of dithiothreitol (DTT; Gibco BRL Products, Gaithersburg, Md.). To provide excess Ca^{2+} or Mg^{2+} ions to assays containing the chelators EDTA and EGTA, either calcium chloride or magnesium chloride (1 or 5 mM final concentration) was added to the reaction cocktails 10 min after the addition of the chelating agent, and the mixture was held at 37°C for a further 10 min, prior to adding the substrate.

Cocultures of *P. ruminicola* B_14 wild-type or mutant strains with gram-positive, ammonia-producing bacteria. To assess the relevance of Gly-Arg-MNAse activity in affecting the rate and extent of ammonia production, the wild-type or mutant strains were cocultured with either *Peptostreptococcus anaerobius* (strain C) or *Clostridium aminophilum* (strain F) by procedures similar to those outlined previously (2). Briefly, the *P. ruminicola* wild-type and mutant strains were grown overnight in defined medium, and the cells were harvested by centrifugation and then resuspended in fresh medium lacking glucose to a cell density equivalent to 400 mg of protein $liter^{-1}$. The resuspended cells were then inoculated with cultures of either strain C, strain F, or sterile medium, at a rate of 10% (vol/vol). Monocultures of all organisms were also established by using the same inoculation rates described above. Samples (1.2 ml) were collected anaerobically at 0, 12, 24, 48, and 72 h postinoculation, and the ammonia concentration in filtered samples was determined colorimetrically by the phenol-hypochlorite method, adapted for use with an AutoAnalyzer II (Technicon Instruments Corp., Terrytown, N.Y.). A second series of experiments, with the inclusion of 0.1% (wt/vol) glucose in the medium, were also conducted.

RESULTS

Survival, mutation frequency, and isolation of Gly-Arg-MNAse-deficient mutants. The survival curves of *P. ruminicola* B_14 following exposure to EMS for various time periods are shown in Fig. 1. Initial viable cell density influenced the efficacy of EMS. When the initial number of viable cells at time zero was 2×10^8 ml^{-1}, the survival curve more closely resembled that characteristic of enteric bacteria (15). However, at both cell densities, treatment with EMS for periods of 30 to 45 min reduced the number of viable cells by at least 40%, and treatment for 60 min reduced viability to 33% of untreated cells. Once the cells were washed to remove EMS and incubated overnight in defined medium, the OD_{600} of the mutagenized cultures increased, but only slightly (<0.1 absorbance unit), suggesting limited outgrowth of the cultures. Samples of these overnight cultures were then tested for the number of Rif^r colonies, and the mutation frequency is shown in Fig. 2. Relative to that of cells not treated with EMS, the frequency of Rif^r colonies increased in response to EMS treatment. Maximal mutation frequencies were achieved by exposure to EMS

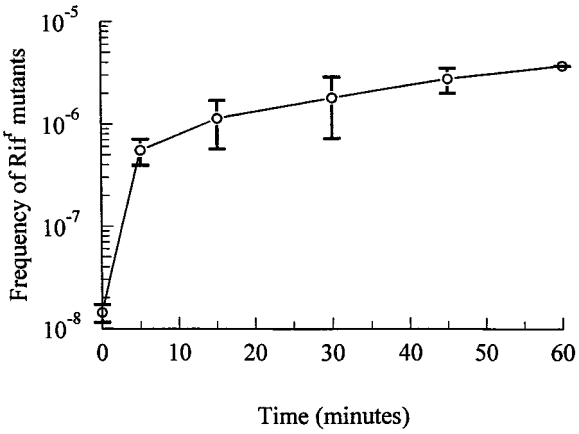

FIG. 2. Plot of rifampin-resistant mutant frequency in *P. ruminicola* B_14, relative to time of exposure to EMS. Mutant frequency was estimated from overnight cultures of EMS-treated cells, with appropriate dilutions spread on either EM plates plus rifampin (20 μg ml^{-1}) or EM plates without antibiotic. The value at time zero represents the rate of spontaneous mutagenesis, and the values at each time point are the averages of results obtained for both cell densities (see Fig. 1). Vertical bars represent standard errors of the means.

TABLE 1. Gly-Arg-MNAse specific activities and doubling times of *P. ruminicola* B_14 wild type and Gly-Arg-MNAse mutant strains GM4 and GM6 following growth with different nitrogen sources

Strain	Gly-Arg-MNAse sp act[a] with N source of:			
	10 mM Ammonia	1.25% Peptides	Ammonia + peptides	Gelatin
Wild type	6.7 (65)	9.3 (67)	7.0 (68)	6.2 (108)
Mutant GM4	0.7 (68)	0.7 (69)	0.6 (67)	0.6 (110)
Mutant GM6	0.7 (67)	0.8 (69)	0.7 (66)	0.6 (101)

[a] Peptidase specific activities (expressed as nanomoles of MNA released per minute per milligram of protein) are the average of four observations obtained from duplicate samples taken from two different cultures. The coefficient of variation for all mean values did not exceed 10%. Doubling times (expressed in minutes and shown in parentheses) are estimated from the semilogarithmic plot of growth curve data collected for two different cultures.

TABLE 2. Peptidase activity in cytoplasmic-periplasmic fractions prepared from *P. ruminicola* B_14 wild-type and Gly-Arg-MNAse mutant strains GM4 and GM6

Peptidase	Sp act[a]		
	Wild type	GM4	GM6
Gly-Arg-MNA	6.70 ± 0.11	0.64 ± 0.01	0.68 ± 0.02
Arg-Arg-MNA	5.30 ± 0.07	0.59 ± 0.02	0.61 ± 0.02
Gly-Pro-MNA	1.00 ± 0.04	1.00 ± 0.09	1.20 ± 0.08
Lys-Ala-MNA	2.20 ± 0.07	1.90 ± 0.11	1.90 ± 0.07

[a] Specific activity is expressed as nanomoles of MNA released per minute per milligram of protein; results are averages of duplicate observations from at least two different cultures ± standard errors of the means.

for periods of 30 min or longer and were approximately 200-fold higher than the frequency of spontaneous mutation to Rif^r. Therefore, we chose to screen for Gly-Arg-MNAse-defective mutants from the culture exposed to EMS for 45 min. Approximately 9,000 colonies were screened for Gly-Arg-MNAse activity by the agarose overlay method, and of these, 15 putative Gly-Arg-MNAse-deficient colonies were picked and restreaked for more direct quantification of Gly-Arg-MNAse activity. Only two of these were found to have reduced Gly-Arg-MNAse activity, possessing 1/10 the specific activity of the wild-type parent strain (Table 1), and are referred to as GM4 and GM6. Both GM4 and GM6 failed to grow under aerobic conditions and were also resistant to chloramphenicol (10 $\mu g\ ml^{-1}$) when that was included in the growth medium. Cell morphology was also typical of that of the wild-type parent strain, and there were no discernible differences in the one-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis protein profiles of the wild-type parent and mutant strains (data not shown). Based upon these findings, we conclude that GM4 and GM6 are, in fact, mutants of *P. ruminicola* B_14. Although growth with Trypticase alone resulted in a small but consistent increase in Gly-Arg-MNAse activity in the wild-type parent strain, enzyme activity remained low in both GM4 and GM6, irrespective of the N source available for growth. The growth rates for the wild-type and mutant strains with different N sources as well as Gly-Arg-MNAse specific activities are shown in Table 1. Despite the virtual elimination of Gly-Arg-MNAse activity in strains GM4 and GM6, their growth rates were very similar to that of the wild-type strain for all N sources tested. Additionally, the final OD_{600} values of the wild-type and mutant cultures were all in excess of 1.0, suggesting that there were minimal differences in cell yield (data not shown). Both mutant strains have also proved to be very stable, being transferred regularly with no alteration in phenotype.

Approximately 90% of total Gly-Arg-MNAse activity in the wild-type strain was present in the cytoplasmic-periplasmic fraction, and the 10-fold reduction in Gly-Arg-MNAse activity for both mutant strains was still apparent after cell fractionation (Table 2). Virtually no Gly-Arg-MNAse activity was measurable in the cell-free culture fluids of wild-type, GM4, and GM6 cultures grown to the mid-log or stationary phase, suggesting that Gly-Arg-MNAse activity remains cell associated at all stages of growth, and the mutant phenotype cannot be simply explained by the release of enzyme activity into extracellular fluids. As such, we interpret these results as showing that the mutated allele(s) in GM4 and GM6 affects either gene expression or enzyme activity rather than the cellular location of the enzyme. A comparison of the wild-type, GM4, and GM6 peptidase enzyme profiles by using several other MNA-conjugated dipeptides is also shown in Table 2. Preliminary experiments with the wild-type strain showed that enzyme activity specific for these different substrates was also present in the cytoplasmic-periplasmic fractions and that stage of growth or nitrogen source did not greatly alter their specific activity (data not shown). Therefore, only the results for the cytoplasmic-periplasmic fractions of cells harvested during the mid-log phase of growth are shown. In addition to the loss of Gly-Arg-MNAse activity, Arg-Arg-MNAse activity in both mutants was also reduced to approximately 1/10 of the levels found in the wild type, while activities against Gly-Pro-MNA and Lys-Ala-MNA were not affected. Moreover, when either whole cells or cell fractions prepared from wild-type cultures were treated with various proteinase inhibitors, the inhibition profile was virtually identical when either Gly-Arg-MNA or Arg-Arg-MNA was used as a substrate (Table 3). Enzyme activity was unaffected by the serine proteinase inhibitor PMSF but was strongly inhibited by cysteine proteinase inhibitors (pCMB and IAA) as well as EDTA and EGTA. Interestingly, enzyme activity could be restored by adding a molar excess of $CaCl_2$ to the reaction mixture, but $MgCl_2$ did not give rise to similar results. Enzyme activity was also greatly reduced when the assays were conducted under aerobic conditions. The addition of DTT to enzyme assays conducted under anaerobic

TABLE 3. Effect of various proteinase inhibitors upon Gly-Arg-MNAse and Arg-Arg-MNAse activities of *P. ruminicola* B_14 wild-type strain

Inhibitor	Enzyme activity[a]	
	Gly-Arg-MNAse	Arg-Arg-MNAse
Control	6.50 (100)	5.10 (100)
PMSF (1 mM)	6.30 (97)	4.80 (94)
pCMB (1 mM)	0.54 (8)	0.53 (10)
IAA (1 mM)	0.65 (10)	0.65 (13)
EDTA (1 mM)	1.13 (17)	1.04 (20)
EDTA + 5 mM Ca^{2+}	5.70 (88)	5.20 (102)
EDTA + 5 mM Mg^{2+}	1.34 (21)	1.07 (21)
EGTA (1 mM)	0.52 (8)	0.55 (11)
EGTA + 5 mM Ca^{2+}	6.00 (92)	4.50 (88)
EGTA + 5 mM Mg^{2+}	1.36 (21)	1.07 (21)
O_2 (aerobic)	0.45 (7)	0.42 (8)
DTT (5 mM)	6.42 (99)	5.00 (98)

[a] Activity is expressed as nanomoles of free MNA released per minute per milligram of protein. Values in parentheses represent percentages of enzyme activity relative to control assays (no additions). Values are the means of duplicate analyses from at least two different cultures. The coefficients of variation for all values did not exceed 10%.

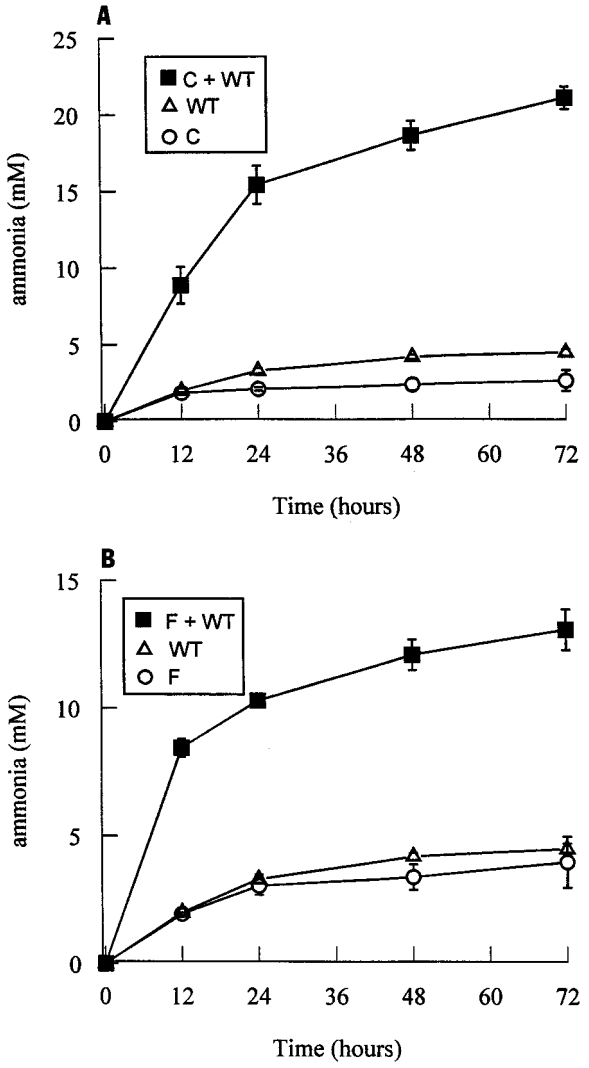

FIG. 3. Ammonia production by monocultures or cocultures of *P. ruminicola* wild-type strain B_14 (WT) and *Peptostreptococcus anaerobius* (strain C) (A) or *Clostridium aminophilum* (strain F) (B) during growth in the presence of gelatin hydrolysate. Ammonia concentrations are corrected for the amount present in the medium prior to inoculation. The values for each time point are the averages of values obtained from two experiments, with each sample being assayed in duplicate. Vertical bars represent standard errors of the means.

conditions had no stimulatory or inhibitory effect on enzyme activity. However, when 2 mM DTT was added to cell suspensions prior to cell disruption, and enzyme assays were subsequently conducted under aerobic conditions, approximately 50% of the activity measured in the control assays was retained. This effect of DTT was only transient because after several days of storage, no measurable enzyme activity remained, and it could not be restored by additional DTT.

Ammonia production kinetics by cocultures. When wild-type *P. ruminicola* B_14 cells were cocultured with either *C. aminophilum* or *Peptostreptococcus anaerobius*, there was a more than additive increase in the rate and extent of ammonia production from gelatin hydrolysate, when compared with that of the respective monocultures (Fig. 3). Cocultures of *P. ruminicola* mutant GM4 or GM6 with either *C. aminophilum* or *Peptostreptococcus anaerobius* also resulted in increased ammonia production from gelatin hydrolysate. However, both the rate and extent of ammonia production were reduced by approximately 25% when compared with those of the wild type (Fig. 4), and the results for both mutants were similar. These decreases in ammonia production in the cocultures cannot be accounted for by the reduction in ammonia production by the mutant strain alone, because these reductions amounted to less than 2 mM. After 72 h of incubation in the presence of gelatin hydrolysate, ammonia concentrations in culture fluids were 4.4, 2.9, and 3.2 mM for the wild-type, GM4, and GM6 strains, respectively. When 0.1% (wt/vol) glucose was included in the medium, overall ammonia production was slightly elevated, but the patterns of ammonia production by the different cocultures were similar to that observed in the absence of glucose (data not shown).

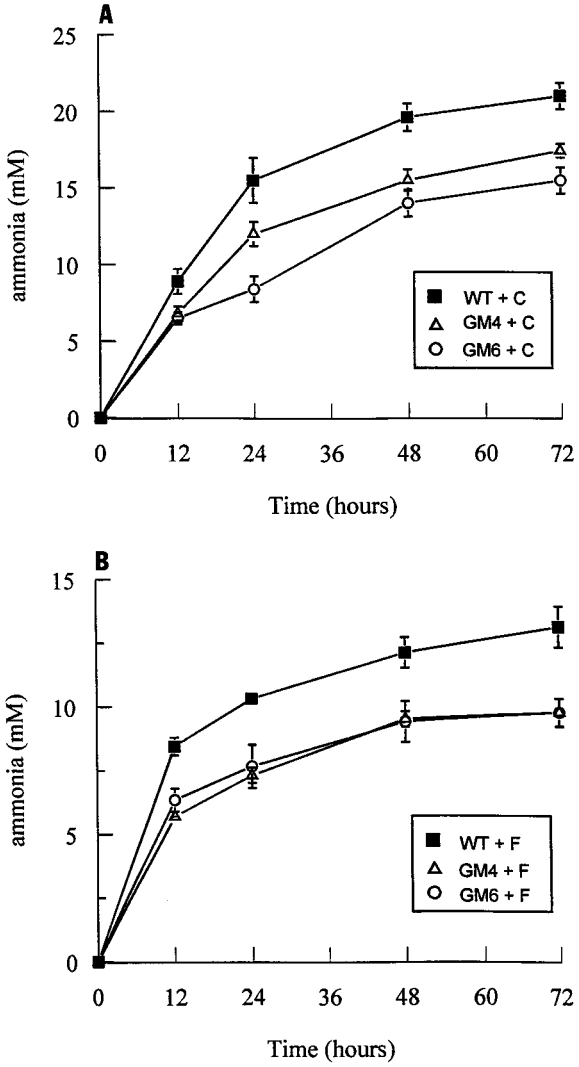

FIG. 4. Ammonia production from gelatin hydrolysate by *Peptostreptococcus anaerobius* (strain C) (A) and *Clostridium aminophilum* (strain F) (B) either as monocultures or during cocultivation with either *P. ruminicola* wild-type strain B_14 (WT) or *P. ruminicola* Gly-Arg-MNAse-defective mutants GM4 and GM6. Ammonia concentrations are corrected for the amount present in the medium prior to inoculation. The values obtained for each time point are the average values obtained from two experiments, with each sample being assayed in duplicate. Vertical bars represent standard errors of the means.

DISCUSSION

Mutagenesis procedures, despite being used extensively in microbiological studies, have found little application to date in the field of ruminal microbiology (7), and to our knowledge, this paper reports the first successful use of chemical mutagenesis with ruminal microorganisms. Newbold et al. (16) described the properties of ionophore-resistant *P. ruminicola* cells enriched for by cultivation in the presence of increasing concentrations of tetronasin; spontaneous mutants of *P. ruminicola* $B_1$4 resistant to rifampin have also been isolated in a similar manner. However, the rate of spontaneous mutagenesis is sufficiently low that without a strong selection strategy (e.g., antibiotic resistance), the isolation of mutants defective in certain enzymes or metabolic pathways is impractical. In the studies reported here, a conventional technique and mutagen were used and the mutation frequency, although lower than that typically found in enteric bacteria (15), reduced the number of colonies to be screened to a feasible number. We have since combined the EMS protocol with ampicillin enrichment procedures to isolate *P. ruminicola* mutants which grow poorly with peptides as a sole N source (19). Although recent advances have been made in the development of gene transfer protocols for use with *P. ruminicola* (5, 24), there are still limitations in terms of gene transfer frequency and available selective markers to facilitate the use of transposon mutagenesis with these bacteria. In the meantime, the straightforwardness of this procedure, allied with the ability to generate stable mutants at reasonable frequencies, makes this technique a useful starting point for a mutational analysis of *P. ruminicola* physiology.

The mutants generated in this work provided useful information regarding one of several diaminopeptidyl peptidases present in *P. ruminicola*. The Gly-Arg-MNAse activity was first identified in ruminal contents by Wallace and McKain (25) and was deemed to be a dipeptidyl peptidase I (DPP I) enzyme by use of a nomenclature system developed for mammalian proteases (13). In our studies, not only was Gly-Arg-MNAse activity virtually eliminated in both mutants but so was the ability to cleave Arg-Arg-MNA. Such results indicate that either one enzyme is responsible for both activities or the genes encoding the respective enzymes are closely linked and that the mutated allele(s) in GM4 and GM6 has a polar (or pleiotropic) effect upon the gene encoding Arg-Arg-MNAse activity. The inhibition profiles for the respective enzyme activities lead us to conclude that the same enzyme is responsible for both activities. Given that the mammalian DPP I enzymes do not hydrolyze Arg-Arg-MNA (14), and Arg-Arg-MNA is the diagnostic substrate for DPP III enzymes (13), we feel that mammalian enzyme nomenclature should be avoided and reference to this enzyme activity should currently be limited to Gly-Arg-MNAse. Interestingly, the inhibition profile of the Gly-Arg-MNAse is strikingly similar to that of gingipain, an extracellular, trypsin-like enzyme isolated from *Porphyromonas* (*Bacteroides*) *gingivalis* that requires cysteine for activation and calcium for stabilization (3). A second cysteine proteinase from *Porphyromonas gingivalis* termed argingipain, which presented a narrow specificity for synthetic substrates containing Arg in the P1 site and hydrophobic amino acids in the P2 and P3 sites, has also been described (8). However, subsequent studies showed gingipain and argingipain to be the same enzyme (18). The strong peptidase activity of *Porphyromonas gingivalis* that in early surveys was referred to as trypsin-like is now known to be associated with this calcium-dependent cysteine proteinase(s) (3, 8, 10, 23). The similarity of the previous results obtained with *Porphyromonas gingivalis* and our findings to date with *P. ruminicola* suggest that this family of cysteine proteinases is not uncommon among bacteria belonging to the *Bacteroidaceae* but has not been identified in other eubacterial lines of descent. As such, further investigation of the genetics and biochemistry of such enzyme activity may contribute to our understanding of the evolution and structure-function of cysteine proteinases.

Bacteria currently classified as *P. ruminicola* have long been recognized for their inability to utilize free amino acids or dipeptides as a nitrogen source, requiring either ammonia or oligopeptides for growth (20). More recent work with *P. ruminicola* $B_1$4 has shown that dipeptides accumulate in culture fluids when the bacterium is cultivated with either casein protein or soy protein as a nitrogen source (6). The Gly-Arg-MNAse enzyme of *P. ruminicola* $B_1$4 (as well as Arg-Arg-MNAse, Gly-Pro-MNAse, and Lys-Ala-MNAse enzyme activities) appear to be located in the cytoplasm-periplasm, but further distinction between these intracellular compartments has to date been precluded by the sensitivity of the enzymes to aerobic conditions. One may hypothesize that the generation of dipeptides by an intracellularly located dipeptidase(s) could support a dipeptide efflux system, which has been shown to exist in other bacteria such as lactococci (9). However, the growth rates of both mutants in batch culture with either Trypticase or gelatin hydrolysate were no different from those observed for the wild type. This is not entirely surprising, considering the similarities in the cleavage specificities of trypsin (used to produce Trypticase) and the peptidase under investigation here. Chemostat studies under growth-limiting conditions with other protein sources might be needed to conclusively demonstrate the involvement of this enzyme activity in energy transactions in the cell.

Although the physiological role of Gly-Arg-MNAse enzyme activity in *P. ruminicola* is still not clear, an ecological role for this enzyme activity was clearly demonstrated by the coculture experiments, reflected in the alteration of ammonia production kinetics. Gelatin hydrolysate was chosen as the substrate for these experiments, to ensure that the results were directly comparable to those of earlier studies that established the prominent roles of *C. aminophilum* and *Peptostreptococcus anaerobius* in controlling ruminal ammonia production (2). The decreases in both the rate and extent of ammonia production when mutants GM4 and GM6 replaced the wild-type strain in cocultures showed that the Gly-Arg-MNAse activity of *P. ruminicola* has a relevant role in controlling ruminal ammonia production. Future strategies which specifically inhibit Gly-Arg-MNAse activity should result in productive alterations in ruminal ammonia production from an environmental and economic perspective.

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