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Cloning, Sequencing, and Characterization of a Membrane-Associated *Prevotella ruminicola* B_14 β-Glucosidase with Cellodextrinase and Cyanoglycosidase Activities

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Prevotella ruminicola **B_14 is a gram-negative, anaerobic gastrointestinal bacterium. A 2.4-kbp chromosomal fragment from *P. ruminicola* encoding an 87-kDa aryl-glucosidase (CdxA) with cellodextrinase activity was cloned into *Escherichia coli* DH5α and sequenced. CdxA activity was found predominantly in the membrane fraction of both *P. ruminicola* and *E. coli*, but *P. ruminicola* localized the protein extracellularly while *E. coli* did not. The hydrolase had the highest activity on cellodextrins (3.43 to 4.13 μmol of glucose released min^{-1} mg of $protein^{-1}$) and *p*-nitrophenyl-β-D-glucoside (3.54 μmol min^{-1} mg of $protein^{-1}$). Significant activity (70% of *p*-nitrophenyl-β-D-glucoside activity) was also detected on arbutin and prunasin. Less activity was obtained with cellobiose, amygdalin, or gentiobiose. CdxA attacks cellodextrins from the nonreducing end, releasing glucose units, and appears to be an exo-1,4-β-glucosidase (EC 3.2.1.74) which also is able to attack β-1,6 linkages. Comparison of the deduced amino acid sequence with other glycosyl-hydrolases suggests that this enzyme belongs to family 3 (B. Henrissat, Biochem. J. 280:309–316, 1991). On the basis of this sequence alignment, the catalytic residues are believed to be Asp-275 and Glu-265. This is the first report of a cloned ruminal bacterial enzyme which can cleave cyanogenic plant compounds and which may therefore contribute to cyanide toxicity in ruminants.**

Ruminant animals such as cattle and sheep have evolved to utilize the extensive fermentative abilities of their reticulo-ruminal microflora. This symbiotic relationship gives the ruminant animal access to a variety of herbaceous foodstuffs such as cellulose and hemicellulose which are nutritionally unavailable to monogastric animals. In addition to cellobiose, cellodextrins are a major product of cellulose hydrolysis in the rumen. Ruminal bacteria that can utilize cellodextrins are common. *Prevotella ruminicola* B_14 possesses extracellular cellodextrinase activity (27) and can utilize water-soluble cellodextrins (up to seven glucose units in length) as carbon and energy sources. In this paper, we report the cloning, sequencing, and partial characterization of an 87-kDa membrane-associated exo-β-glucosidase.

Although normally beneficial, the expanded digestive repertoire of the ruminant animal can also be a detriment. A significant number of range forages such as serviceberry (*Amelanchier alnifolia*), bird's foot trefoil (*Lotus corniculatus*), chokeberry (*Prunus virginiana*), various sorghum species, white clover (*Trifolium repens*), and arrowgrass (*Triglochin* spp.) contain cyanogenic glycosides which are hydrolyzed during ruminal fermentation. These glycosides constitute a prominent class of toxic plant compounds (7, 9, 31) which presumably are synthesized to discourage grazing. For a review of the plant biosynthetic routes that produce these glycosides, see the work of Vennesland et al. (31).

The *P. ruminicola* B_14 β-glucosidase catalyzes the first step in cyanide release from cyanogenic plant glycosides. Since *P. ruminicola* can account for 40 to 50% of the total culturable bacteria detected in either pasture-fed cattle (30) or those fed grass silage (30), this activity may play a significant role in cyanide toxicity. To our knowledge, this is the first report of an isolated enzyme from a ruminal bacterium which is capable of cleaving cyanogenic plant compounds. This enzyme released glucose units from aryl-glucosides, cellodextrins, and cyanogenic glycosides but had less activity on cellobiose or gentiobiose. By isolating enzymes responsible for the hydrolysis of both beneficial and toxic plant compounds, the dynamics of ruminal forage digestion under optimal as well as adverse conditions can be explored.

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MATERIALS AND METHODS

Bacterial strains and growth conditions. *P. ruminicola* B_14 (6) was obtained from Terry Miller (Albany, N.Y.). *Escherichia coli* LE392 is described by Sambrook et al. (28). *E. coli* DH5α and *E. coli* DH5αIQ were purchased from GIBCO BRL, (Gaithersburg, Md.). *P. ruminicola* was grown under anaerobic conditions at 39°C as described previously (23), with 0.3% cellobiose as the carbon and energy source. *E. coli* LE392 was grown at 37°C with aeration in Luria-Bertani (LB) broth (28) supplemented with 0.2% maltose to induce the maltose permease. *E. coli* DH5α and DH5αIQ were grown at 37°C with aeration in LB broth or solid LB medium containing 1.5% agar. Ampicillin was added when needed at a final concentration of 100 μg ml^{-1}.

DNA techniques and cloning. All λ phage manipulations were performed as previously described (28), and the multiplicity of infection used was 0.04. Plasmid DNA was extracted from *E. coli* hosts by using an alkaline lysis method (28) or the Plasmid-Select plasmid purification kit (5′→3′, Boulder, Colo.). Restriction enzymes, buffers, and T4 ligase were purchased from New England Biolabs (Beverly, Mass.) or United States Biochemical (Cleveland, Ohio). Unless otherwise indicated, restriction enzyme digestions and subsequent ligations were performed according to the manufacturer's recommendations. Individual *P. ruminicola* DNA fragments were isolated on agarose gels and purified by using the Gene Clean II kit (Bio 101, La Jolla, Calif.).

Plasmid constructs were introduced into washed *E. coli* DH5α cells by electroporation (Gene Pulser; Bio-Rad, Hercules, Calif.). Positive recombinants were detected either on LB-ampicillin plates containing 0.04% 5-bromo-4-chloro-3-indolyl-β-D-glucopyranoside (X-Glu; Sigma, St. Louis, Mo.) or on LB-

ampicillin plates overlaid with top agar containing 0.04% 4-methylumbelliferyl-β-D-glucoside (MUG; Sigma) dissolved in dimethylformamide. Colony fluorescence was detected with a transilluminator (Fotodyne, New Berlin, Wis.). $CaCl_2$-treated *E. coli* DH5αIQ cells were transformed with recombinant M13 phage, plated, and detected as described in the Bethesda Research Laboratories manual (4).

Total *P. ruminicola* $B_1$4 chromosomal DNA was digested with *Eco*RI, packaged into λ2001 phage, and screened with X-Glu as described above (23). Standard molecular cloning techniques were used (28). Partial digestion of the positive phage λWS8 with *Hin*dIII resulted in an 11-kb *P. ruminicola* fragment which was ligated to pUC19, to produce pWS2. Plasmid pWS2 was then partially digested with *Sau*3AI, and the DNA fragments were separated by size, using a sucrose density gradient. Fragments of 4 to 6 kb in size were ligated to *Bam*HI-digested pBluescript SK^+, transformed into *E. coli*, and screened for activity. Plasmid pWS21, which was constructed in this manner, contained a 4-kb insert. *Hin*dIII digestion of pWS21 and ligation under dilute conditions resulted in a 1.1-kb deletion to produce pWS22, which contained a 2.9-kb *Bam*HI-*Hin*dIII insert. The plasmids and bacteriophage used in this study are summarized in Fig. 1.

DNA sequencing. Three recombinant M13 phages were constructed for DNA sequencing. M13ϕ1 contained the entire 3-kb *Hin*dIII-*Bam*HI insert of pWS22 in M13mp19. A 2.2-kb *Pst*I-*Bam*HI fragment from pWS21 was oriented in the opposite direction in M13mp18, to produce M13ϕ4. A 1.8-kb *Hin*dIII-*Pst*I fragment derived from pWS21 and ligated to M13mp19 resulted in M13ϕ3.

Single-stranded DNA was purified from recombinant M13 phage as described in the Bethesda Research Laboratories manual (4). Plasmid DNA for double-stranded DNA sequencing was purified as described above. Double-stranded DNA was denatured by boiling for 5 min prior to annealing. DNA sequencing was carried out on either single- or double-stranded DNA with T7 Sequenase obtained from United States Biochemical, according to the Sequenase protocol. Manual sequencing results were verified by automated sequencing performed on a 373A DNA Sequencer (Applied Biosystems Inc., Foster City, Calif.). Specific synthetic oligonucleotide primers 17 to 21 nucleotides in length (produced by the Cornell University Biotechnology Program Oligonucleotide Synthesis Facility) were used to sequence along DNA fragments.

Southern hybridizations. Recombinant, replicative-form M13 DNA from infected *E. coli* cells was purified and digested with appropriate restriction enzymes. The fragments were separated electrophoretically in 0.8% agarose and transferred to nitrocellulose filters (28). Southern hybridizations with digoxigenin-labeled probes and subsequent alkaline phosphate detection of positive hybrids were performed according to the Genius System protocol (Boehringer Mannheim, Indianapolis, Ind.).

Cellular fractionation. *P. ruminicola* $B_1$4 and *E. coli* WS21 were grown in liquid media as described above. All techniques were performed at 4°C unless otherwise noted. The cultures were harvested by centrifugation, washed with 50 mM 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer, pH 6.0, and resuspended with MES buffer to 5% of the original volume. An aliquot from each culture was saved and designated whole cells. The resuspended cells were disrupted with a French pressure cell at 20,000 lb/in^2, and phenylmethylsulfonyl fluoride was added to a final concentration of 0.1 mM. The extent of breakage was assessed microscopically by visual inspection. An aliquot from each culture was removed and designated broken cells. Cellular debris was removed by centrifugation (13,000 × *g* for 10 min). Nucleic acid polymers were removed by DNase and RNase digestion at 37°C for 1 h (5 μg/ml each; Sigma). An aliquot from each suspension was removed and designated crude cell extract. The resultant crude cell extract was subjected to ultracentrifugation (100,000 × *g*, 1 h) to pellet the membranous fraction. The soluble fraction was designated cytoplasmic proteins. The membrane pellet was resuspended in 2 ml of 50 mM MES buffer, pH 6.0. Samples stored at 4°C were stable for up to 2 weeks (data not shown). Enzymatic assays were performed within 2 days of harvest. Protein concentrations were determined by the method of Lowry et al. (18). Cellular fractions were boiled for 20 min in 0.2 N NaOH prior to protein analysis.

Membrane preparations. Overnight cultures of *E. coli* WS21 were harvested by centrifugation at 6,800 × *g* for 15 min, washed, and resuspended to 5% of the original culture volume in 50 mM MES buffer, pH 6.0. The concentrated cells were broken by passage through a French pressure cell at 18,000 lb/in^2 and diluted with MES buffer to 1/10 the original culture volume, and phenylmethylsulfonyl fluoride was added to a final concentration of 0.1 mM. Prior to the removal of large debris by centrifugation, NaCl was added to 0.5 M to maximize the solubility of small membrane fragments. Unbroken cells and large cellular debris were sedimented by centrifugation at 13,000 × *g* for 10 min. Streptomycin sulfate was added to the supernatant fluid at a final concentration of 4% to precipitate nucleic acids. After a 30-min incubation, the sample was centrifuged at 13,000 × *g* for 10 min. Cytoplasmic proteins were separated from the membranes by centrifugation at 100,000 × *g* for 1 h and discarded. The resultant membrane pellet was resuspended in MES buffer to 1% of the original culture volume and stored at −20°C. Membrane preparations were utilized within 1 week of harvest. The approximate protein concentration of these preparations was 10 mg ml^{-1}.

N-terminal sequence analysis. Polypeptides from membrane preparations of *E. coli* WS21 cells were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The separated proteins were transferred electrophoretically in 10 mM 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS, pH 11; Sigma), dissolved in 10% methanol onto Immobilon-P (Millipore, Bedford, Mass.) nitrocellulose filters with a TE 22 Mighty Small Transphor Tank Transfer Unit (Hoefer Scientific Instruments, San Francisco, Calif.), and briefly stained with Coomassie blue. The location of the cloned cellodextrinase on the filter was identified by visual comparison with extracts of *P. ruminicola* as well as extracts from nonrecombinant *E. coli* DH5α (see Fig. 3). Filter strips containing the protein were excised, dissolved in propionic acid, and subjected to automated Edman degradations with a Gas Phase Protein Sequencer (model 470A; Applied Biosystems Inc.) equipped with a 120A PTH analyzer.

Antibody production and Western blotting (immunoblotting). The cloned cellodextrinase was separated from total membrane proteins by SDS-PAGE (see Fig. 3). Washed and homogenized gel slices were used to obtain polyclonal rabbit antibodies as described by Harlow and Lane (11). The presence of antibodies to the CdxA protein was verified by using broken cells of *P. ruminicola* and membrane extracts of *E. coli* WS21 and *E. coli* D571. Proteins were separated on SDS-polyacrylamide gels and electrophoretically transferred to nitrocellulose membranes (BA85; Schleicher and Schuell) as described above. Goat anti-rabbit immunoglobulin G–alkaline phosphatase conjugate (Bio-Rad, Melville, N.Y.) was used as the second antibody and detected as described by the supplier.

Enzyme assays. Aryl-glucosidase activity was measured with *p*-nitrophenyl-β-D-glucoside (PNPG; Sigma). The assays were carried out as follows. Crude cell extract (10 μl) (see above) was added to 720 μl of 50 mM MES buffer, pH 6.0, and allowed to equilibrate to 39°C for 5 min. The reaction was started by the addition of 20 μl of 100 mM PNPG dissolved in MES buffer and allowed to proceed for timed intervals at 39°C. Enzyme activity was terminated by the addition of 250 μl of 2 M Na_2CO_3, pH 11, and A_{401} was measured immediately. The reported extinction coefficient for *p*-nitrophenol at this pH is 18.5 mM^{-1} (Sigma).

Arbutin hydrolysis was measured by the release of hydroquinone according to the method of Oglesby et al. (24). CdxA reactions were run in a total volume of 800 μl as described above; however, enzyme activity was terminated by the addition of 700 μl of isopropyl alcohol. Phloroglucinol (70 μl, 5% solution) and KOH (70 μl, 1 N) were slowly added to each tube; the tubes were gently shaken and incubated for 1 h in the dark at room temperature. The A_{520} was measured and compared with a hydroquinone standard curve.

The hydrolysis of cellodextrins, cellobiose, or gentiobiose was measured by glucose production. In a total volume of 400 μl, an appropriate volume of crude cell extract was equilibrated in MES buffer to 39°C as described above. At timed intervals, the reactions were terminated with the addition of 100 μl of 2 M Na_2CO_3, pH 11, and neutralized with 50 μl of 6 M HCl. Neutralization did not restore CdxA activity (data not shown). Glucose assays were performed according to the method of Bergmeyer and Klotsch (3) with the following modifications. A reaction mixture (450 μl) containing 100 mM triethanolamine buffer (pH 7.5), 8 mM $MgCl_2 \cdot 6H_2O$, 1.5 mM ATP, 1 mM NADP, hexokinase (9 U; Sigma), and glucose-6-phosphate dehydrogenase (12.6 U; Sigma) was added to each tube. The reduction of NADP to NADPH was monitored at 340 nm and compared with a similarly treated glucose standard curve.

The hydrolysis of amygdalin or prunasin was monitored by high-pressure liquid chromatography (HPLC). *E. coli* WS21 crude cell extract was incubated in 50 mM MES buffer, pH 6.0, with 50 mM (either) amygdalin or prunasin at 39°C in a total volume of 1,200 μl. At 2-h intervals, reaction samples were removed and the reactions were terminated by freezing the samples in −80°C ethanol. The products were separated by using a Dynamax -NH_2 60Å column (Rainin Instruments, Woburn, Mass.). A solvent consisting of 65% acetonitrile and 35% water at a flow rate of 0.75 ml/min was used to elute the samples, which had an average product recovery of 91.5%. Hydrolysis rates were calculated from the products obtained from the 2-h reaction incubations as determined by HPLC.

Assays were performed as described by Kotzé (15) with 50 mM PIPES [piperazine-*N*-*N'*-bis(2-ethanesulfonic acid)], pH 7.0, substituted for 100 mM Tris buffer.

Computer analysis. Computer analyses of DNA and protein sequences were performed with the DNA Inspector IIe program (Textco Inc., West Lebanon, N.H.) and the Genetics Computer Group software package, version 7.3 (Genetics Computer Group Inc., Madison, Wis.).

Nucleotide sequence accession number. The nucleotide and amino acid sequences of *cdxA* were submitted to GenBank under accession number U35425.

RESULTS

Cloning and sequencing of the *P. ruminicola cdxA* gene. *P. ruminicola* has considerable cellodextrinase activity (27). To isolate the gene responsible for this activity, we cloned an 11-kb fragment from a previously prepared *P. ruminicola* chromosomal library (23) which gave a positive signal on X-Glu plates into pUC19 to produce WS2. Subcloning the insert from pWS2 (as described in Materials and Methods) resulted in several smaller plasmids which conferred X-Glu hydrolysis activity to *E. coli*. Recombinant *E. coli* containing plasmid

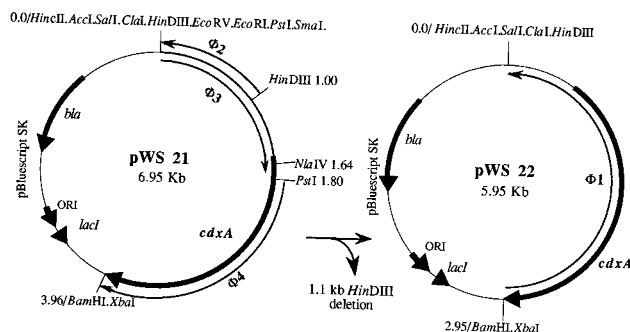

FIG. 1. Diagram of the plasmids used in this study. The *cdxA* gene of *P. ruminicola* $B_1$4 is indicated by a long thick arrow. The thin arrows represent the length, location, and sequencing direction of fragments φ1 through φ4, which were cloned into M13 phage to produce M13φ1 through M13φ4, respectively. (See Materials and Methods for details.)

pWS21 (*E. coli* WS21; Fig. 1) showed the highest cellodextrinase expression. The overall enzyme yield was not enhanced by placing the 4-kb *Sma*I-*Bam*HI fragment behind the *lacZ* promoter. The *lacZ* promoter may have increased the production of cellodextrinase to a deleterious concentration, because the resultant clone grew fivefold more slowly than *E. coli* WS21 and gave consistently poor yields of cell protein (data not shown).

The cellodextrinase gene was sequenced by using M13 bacteriophage clones. The nucleotide sequence of the *P. ruminicola cdxA* gene is presented in Fig. 2. A 3.1-kb *Bam*HI-*Hin*dIII fragment containing the entire *cdxA* gene (Fig. 1) was cloned into M13mp19 and designated M13φ1. We could not recover the same insert in M13mp18. Reversal of the intact gene's orientation in M13 may have permitted expression of the cellodextrinase, which in conjunction with M13 infection resulted in lethality. To avoid this possible problem, degenerate DNA sequences derived from the cloned protein's N-terminal amino acid sequence (see below) were used to identify the location and orientation of *cdxA* within the cloned inserts. A separate phage (M13φ3) was then constructed to sequence *cdxA* DNA in the forward direction as well as its upstream regions. Deletion of the first 225 nucleotides of *cdxA* to produce M13φ4 was sufficient to allow recovery of the rest of the gene in the forward orientation in M13. All recombinant phage were confirmed by Southern hybridization to digoxigenin-labeled probes which had been synthesized from X-Glu-positive plasmids (data not shown).

Protein characteristics. The determined amino-terminal sequence of the cloned protein is MNKKIGVFALGILLSGA. A putative signal sequence of 22 amino acids (MNKKIGVFAL GILLSGASMAQA) was identified by inspection of the DNA sequence on the basis of typical signal sequences as described by Reithmeier and Deber (26). Similar to the case for the xylanase gene of *P. ruminicola* (33), there is no apparent *E. coli*-like Shine-Dalgarno sequence. The sequence-predicted molecular mass of the cellodextrinase is 86.6 kDa. A prominent band at approximately 87 kDa was observed within SDS-PAGE-separated proteins from *E. coli* WS21 cell extracts (Fig. 3). Western blots of *P. ruminicola* extracts revealed a protein which was slightly smaller than the cloned *E. coli* enzyme, which is consistent with cleavage of the signal sequence in *P. ruminicola* (Fig. 3). The predicted isoelectric point of the cellodextrinase is near neutrality at pH 7.55. Neither the Kyte-Doolittle nor the Hopp-Woods computer algorithm predicted any extended regions of significant hydrophobicity or hydrophilicity (data not shown).

Amino acid sequence comparisons. The CdxA protein sequence was aligned with several members of glycosylhydrolase family 3 (12), using Genetics Computer Group software. The percent identities of these enzymes with CdxA ranged from 27 to 35, suggesting that CdxA was distantly related to other enzymes.

An aspartic acid in the active site of the extensively studied *Aspergillus wentii* β-glucosidase is known to be one of two catalytic residues (1, 16). The other is a glutamic acid 9 residues upstream of the aspartic acid. This enzyme has recently been classified in family 3 (13). Figure 4 shows the amino acid alignment of the active site of the *A. wentii* β-glucosidase to the predicted active sites of several other family 3 β-glucosidases as well as CdxA. In all cases, the predicted aspartic acid is conserved. The glutamic acid is also conserved in all but the *Ruminococcus albus* enzyme. The putative catalytic residues in CdxA are Glu-265 and Asp-275.

Enzyme location. Late-logarithmic-growth-phase cultures of *P. ruminicola* $B_1$4 and *E. coli* WS21 were harvested, lysed, fractionated, and assayed for *p*-nitrophenyl-β-D-glucosidase and aldolase activities. PNPG was cleaved by whole cells of *P. ruminicola* but only minimally by whole-cell suspensions of *E. coli* WS21 (Table 1). For both bacteria, the majority of β-glucosidase activity was found in the crude cell extract; however, activity was also detected in the resuspended cellular debris (see Materials and Methods). In *P. ruminicola*, this activity was a substantial portion of the total activity (25%), while in *E. coli* it represented 5% of the total activity. Prior work has shown that the PNPG hydrolase activity of *P. ruminicola* is not liberated by osmotic shock (29).

Separation of total membranes from crude extracts was accomplished by centrifugation, and there was a twofold increase in aldolase specific activity in the cytosol fraction of both cultures compared with the starting material (Table 1). No aldolase activity was detected in the membrane fraction of either culture. Both *P. ruminicola* and *E. coli* WS21 had significantly more PNPG hydrolase activity in the total membrane fraction than in the cytosol fraction (Table 1). These results indicate that the PNPG hydrolase activity of *P. ruminicola* and *E. coli* WS21 is membrane associated. Despite vigorous attempts at solubilization, including using several detergents (nonionic, Zwittergent, and ionic), varying the micelle and protein concentrations, and using various ionic, pH, reducing, and thermal conditions, solubilization was not achieved (data not shown).

Enzyme activity. The activity and kinetic properties of CdxA were determined with several substrates (Table 2). *E. coli* D571 had no detectable activity on these substrates (data not shown). Although the membrane fraction of *E. coli* WS21 had the highest specific activity on PNPG, crude extract preparations were used for these assays (see Discussion). The substrates were assayed at a reaction concentration of 20 mM, with the exception of amygdalin and prunasin, which were assayed at 50 mM (see below).

CdxA had high activity on PNPG, arbutin, cellotriose, cellotetraose, and cellopentaose (Table 2). However, since cellodextrin hydrolysis was measured as micromoles of glucose produced rather than micromoles of substrate hydrolyzed, the reported cellodextrinase activities may be artificially high (see Discussion). Substantially less activity was observed when cellobiose or gentiobiose was used as the substrate (Table 2). Of the substrates tested, CdxA had the highest affinity for PNPG (0.42 mM). Despite the structural similarity between the two substrates, the K_m for arbutin was 10-fold higher than that of PNPG, although the calculated V_{max} values for PNPG and

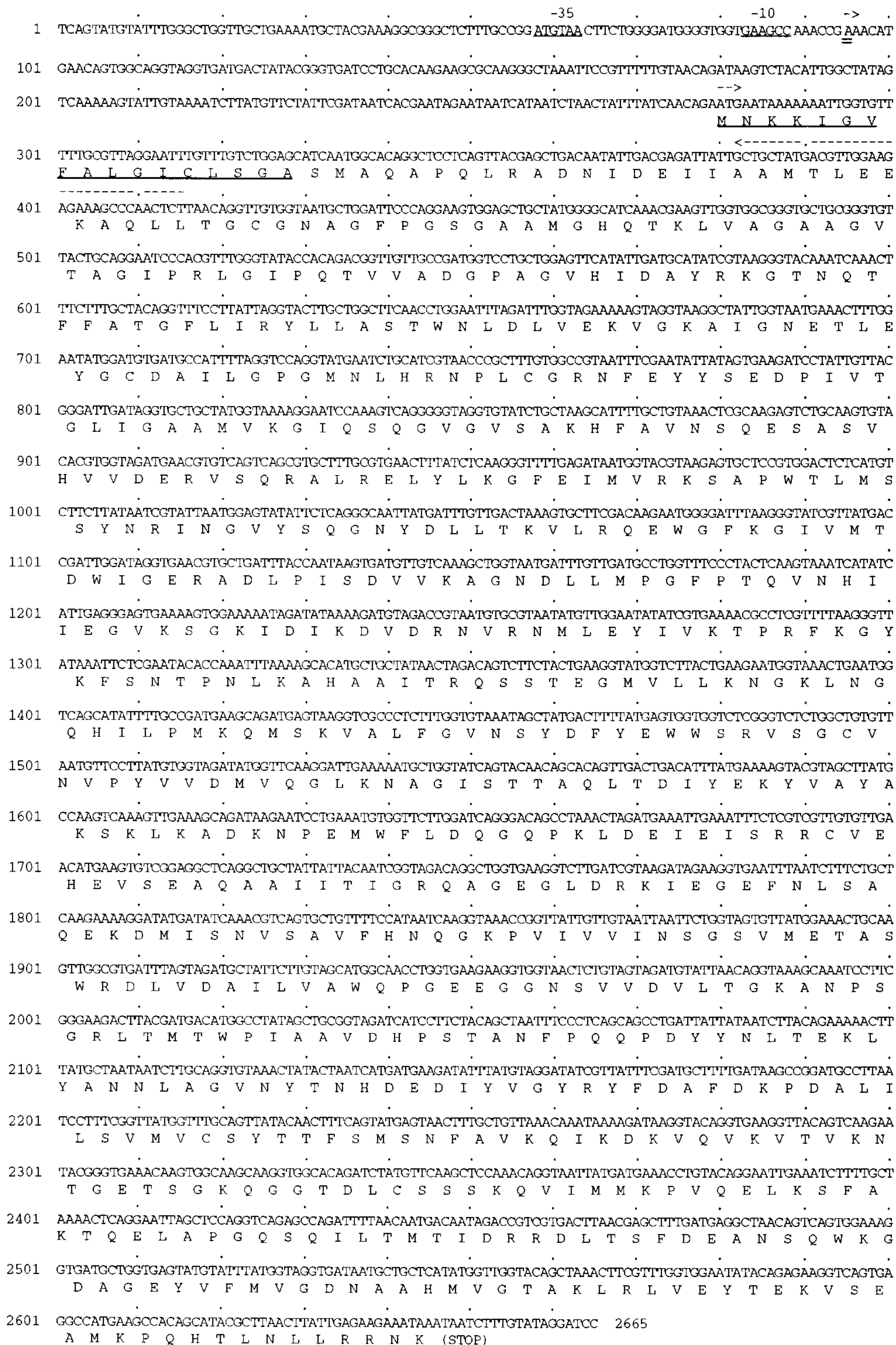

FIG. 2. Nucleotide sequence of *cdxA* from *P. ruminicola* B_14. The predicted amino acid sequence is shown (the one-letter code is used) beneath the nucleotide sequence. The transcriptional start site in *E. coli* is double underlined. The directions of transcription and translation are indicated by the arrows, and the −35 and −10 regions are labeled as such. The 30-mer oligonucleotide used for primer extensions is indicated by a long dashed arrow, while the experimentally determined N-terminal amino acid sequence is underlined.

arbutin were the same (Table 2). Similarly, the calculated V_{max} of CdxA on cellobiose is somewhat lower than that on PNPG (2.5-fold); however, the K_m for cellobiose is approximately 30-fold higher than that for PNPG (Table 2). In contrast, gentiobiose and cellobiose had similar K_m values, while the calculated V_{max} for gentiobiose was the lowest of those of all the substrates tested at 0.35 μmol min^{-1} mg of $protein^{-1}$ (Table 2). Kinetic assays were not performed on purified cellodextrins, because of the potential for mixed substrate-product interferences in the calculations.

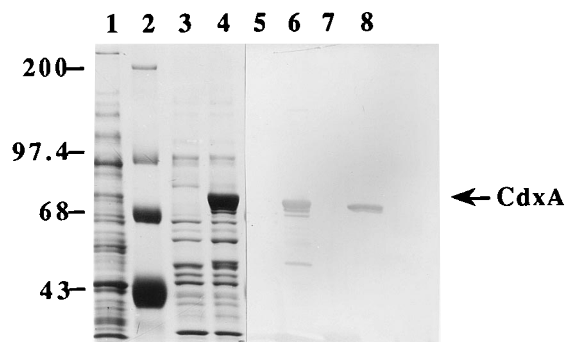

FIG. 3. Identification of the CdxA protein in *P. ruminicola* B_14 and *E. coli* WS21. SDS–8% polyacrylamide gel (lanes 1 to 4) and Western blot with CdxA antibody (lanes 5 to 8) of protein extracts from *P. ruminicola*, *E. coli* WS21, and nonrecombinant *E. coli* DH5α (see Materials and Methods for details). Lanes 1 and 8, total proteins from *P. ruminicola*; lanes 3 and 5, total membrane proteins of *E. coli* DH5α; lanes 4 and 6, total membrane proteins of *E. coli* WS21. (The fainter, low-molecular-weight bands in lane 6 are CdxA proteolytic products.) Lane 2 contains molecular weight markers; lane 7 is blank. The location of CdxA is indicated with an arrow.

Both of the cyanogenic glycosides, amygdalin and prunasin, were cleaved by CdxA (Fig. 5). At a substrate concentration of 50 mM, the specific activity for prunasin was 2.5-fold higher than that for amygdalin (Table 2). Hydrolysis rates for these substrates were calculated with HPLC data.

DISCUSSION

The predicted amino acid sequence of CdxA was compared with other glycosidase sequences. There was similarity to several β-glucosidases which belong to family 3 (12, 13); on the basis of these alignments, we believe that CdxA should be classified in family 3. A membrane location of CdxA is consistent with the fact that most of the enzymes in family 3 are either membrane bound or secreted. It is interesting to note that this family contains several enzymes from other ruminal bacteria as well as β-glucosidases which have characteristics more typical of fungal enzymes than bacterial enzymes (10). Recently, a xylanase gene from *P. ruminicola* 23 was also found to be similar to other bacterial xylanases cloned from various ruminal species (33). These two examples suggest the possible occurrence of genetic exchange within the rumen.

The cellodextrinase appeared to be membrane associated and exposed on the *P. ruminicola* surface, on the basis of the observation that whole cells had the same or even slightly higher activity towards PNPG than disrupted cells. Transport of PNPG into *P. ruminicola* does not appear to be necessary for whole cell activity, because hydrolase activity was indistinguishable from that of lysed cells in the presence or absence of several transport inhibitors (data not shown). An external location for cellodextrinase activity in *P. ruminicola* is also supported by the observations of Russell (27). The lower activity observed in broken cells was probably due to the presence of large membrane fragments which were pelleted by low-speed centrifugation along with 25% of the total activity. These nonordered membranes may have sequestered CdxA, thus hindering substrate availability. Activity was not restored by combining the resuspended pelleted materials with supernatant (data not shown). As a result of this loss and the low solubility of the membranes, activity assays were performed on crude extracts rather than membrane suspensions.

Similarly, enzymatic activity was also membrane associated in *E. coli* WS21; however, cellular disruption was necessary to measure significant activity. This suggests an interior membrane location for the cloned enzyme, which explains why the cloned enzyme retained its signal sequence. Although CdxA was overexpressed 10-fold in *E. coli* relative to expression in *P. ruminicola*, microscopic examination of whole cells, disrupted cells, vesicles, or cell extracts did not reveal the presence of inclusion bodies in *E. coli* WS21.

The type of membrane attachment or insertion of this protein is unknown. Hydrophobicity plots did not reveal any significant hydrophobic regions that would explain the membrane localization. Although uncommon, a lack of significant hydrophobicity has been observed in a few other membrane proteins. An alternate hypothesis is that CdxA is a lipoprotein. Lipoproteins are present in several bacterial species (34), including the extracellular pullulanase in *Klebsiella pneumoniae* (25). A bacterial consensus lipoprotein signal sequence is Leu-Leu-Ala-Gly-Cys-Ser-Ser-Asn-Ala (32), where a glyceryl moiety is posttranslationally attached to the cysteine residue which in turn can be modified to anchor the protein to the bacterial membrane. There are two amino acid sequences at the N terminus of CdxA (Ala-9–Leu-10–Gly-11–Ile-12–Cys-13–Leu-14–Ser-15–Gly-16–Ala-17 and Leu-45–Leu-46–Thr-47–Gly-48–Cys-49–Gly-50–Asn-51–Ala-52–Gly-53) which are very similar to the lipoprotein consensus signal sequence. However, while the length of the hydrophobic core plus the carboxy-terminal length of many lipoprotein signal sequences is on average 14.7 amino acid residues (32), the sums of these lengths for the two CdxA sites are only 8 and 7 residues, respectively.

β-Glucosidases can exhibit high specificity for their natural substrate. CdxA was tested on several naturally occurring sugars to identify its true substrate. The active compounds fall into three categories on the basis of their chemical characteristics: β-1,4-linked, water-soluble cellodextrins (cellotriose, cellotetraose, and cellopentaose); β-linked aryl-glucosides (arbutin and prunasin); and β-1,6-linked glucosides (gentiobiose and amygdalin). The specific activity values of CdxA within each

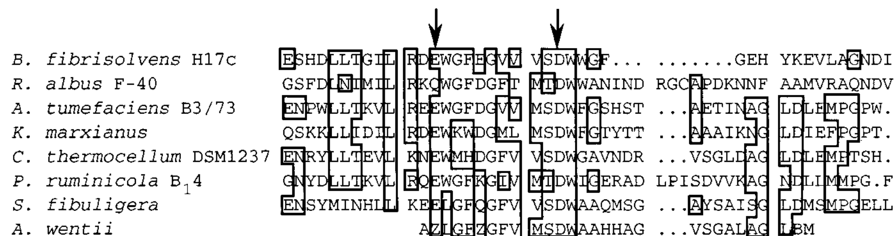

FIG. 4. Amino acid alignment of the β-glucosidase active site from *A. wentii* with other β-glucosidases of glucohydrolase family 3 and CdxA of *P. ruminicola* B_14. Identical residues which are present in four or more proteins are boxed. Vertical arrows indicate presumed active-site catalytic residues. The letter Z represents glutamine or glutamic acid. The letter B represents asparagine or aspartic acid.

TABLE 1. The location of *p*-nitrophenyl-β-D-glucosidase and aldolase activities in different cell fractions of *P. ruminicola* $B_1$4 and *E. coli* WS21

Cell fraction	Total protein (mg)		*p*-Nitrophenyl-β-D-glucosidase Sp act[a]		*p*-Nitrophenyl-β-D-glucosidase Total activity[b]		Aldolase sp act[a]	
	P. ruminicola	*E. coli* WS21	*P. ruminicola*	*E. coli* WS21	*P. ruminicola*	*E. coli* WS21	*P. ruminicola*	*E. coli* WS21
Whole cells	43.2	133.9	1.12	0.14	48.4	18.7	ND[c]	ND
Broken cells	40.6	127.5	0.97	3.38	39.4	431.0	ND	ND
Crude extract	35.5	103.4	0.65	3.39	21.8	350.5	0.05	0.03
Cellular debris	3.9	10.6	2.54	2.07	9.9	21.9	ND	ND
Cytosol	22.3	58.0	0.07	0.08	1.6	4.6	0.09	0.06
Membranes	5.7	18.6	1.64	8.63	9.3	160.5	<0.0001	<0.0001

[a] Micromoles per minute per milligram of protein.
[b] Micromoles per minute.
[c] ND, not determined.

group were similar. The highest enzymatic rates were obtained when cellodextrins were used as the substrates for CdxA; the next highest rates were obtained with the aryl β-glucosides and, finally, the β-1,6-glucosides. Cellodextrin hydrolysis is reported in micromoles of glucose released minute^{-1} milligram of protein^{-1}, because the cellodextrin hydrolysis products are also potential substrates for CdxA. However, since the average amount of glucose produced during the incubation time allotted was 0.001% of the starting cellodextrin substrate concentration and the cellobiose hydrolysis rate was low, it is unlikely that the cellodextrin products were further reduced to glucose. The low activity seen on cellobiose is mainly due to its high apparent K_m.

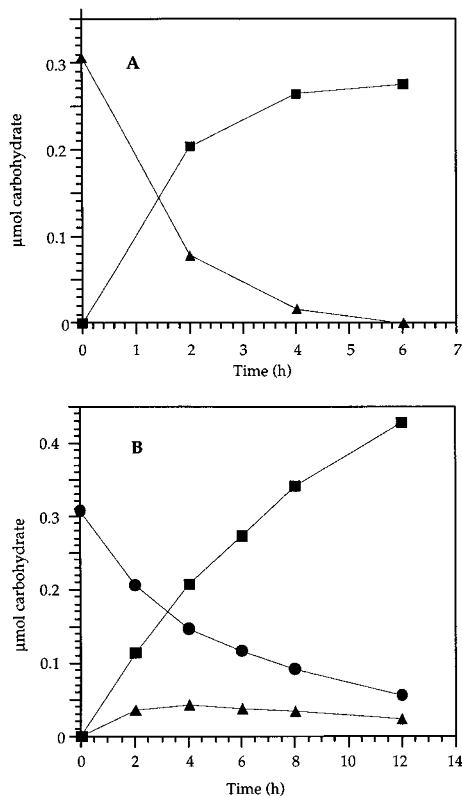

FIG. 5. The products of prunasin and amygdalin hydrolysis by CdxA over time. (A) Prunasin (▲) hydrolysis with production of glucose (■). (B) Amygdalin (●) hydrolysis with release of prunasin (▲) and glucose (■).

The directionality of CdxA attack was determined by using cellodextrins which were reduced by sodium borohydride treatment. CdxA was incubated with these cellohexitols, and the hydrolysis products were separated by thin-layer chromatography. No glucitol (sorbitol) was produced from cellotriositol, cellotetraositol, or cellopentaositol, which would be expected if the direction of cleavage were from the reducing end (data not shown). These experiments indicated that CdxA attacks cellodextrins from the nonreducing end to produce successive glucose units. This result, in addition to the presence of an internal cellobiose phosphorylase in *P. ruminicola* (17) and the low activity seen with cellobiose as the substrate, suggests that CdxA is not involved in cellobiose utilization by *P. ruminicola*. Instead, the normal physiological role of CdxA probably involves cellodextrin or β-glucan hydrolyses, and thus, CdxA is a 1,4-β-D-glucan glucohydrolase (EC 3.2.1.74).

Ruminants are particularly susceptible to cyanide poisoning (5, 8). In the 1980s Majak and Cheng (19, 20) demonstrated that many ruminal bacteria, including *P. ruminicola*, have the ability to release free cyanide from the glycosides amygdalin (laetrile), prunasin, and linamarin. β-Glucosidases play a key role in the initial step of hydrolyzing these compounds into glucose units and the cyanogenic aglycone (8, 22). At pH levels normally found in the rumen (pH > 6), subsequent dissociation of free HCN from the cyanohydrin is rapid (21). The resultant cyanide is then able to enter the animal's blood supply via diffusion across the rumen wall (5, 8). Among range-fed

TABLE 2. The activities of extracts of *E. coli* WS21 on several substrates

Substrate[a]	K_m (mM)	V_{max}[b]	Sp act[b]	Relative activity (%)
PNPG	0.42	3.83	3.54	100
Amygdalin	ND[c]	ND	1.13[d]	32
Arbutin	5.00	3.49	2.47	70
Cellobiose	13.50	1.53	0.93	26
Cellopentaose	ND	ND	3.44[e]	103
Cellotetraose	ND	ND	4.13[e]	117
Cellotriose	ND	ND	3.43[e]	97
Gentiobiose	10.70	0.35	0.23	7
Prunasin	ND	ND	2.53[d]	72

[a] All substrates were present at 20 mM, except amygdalin and prunasin, which were present at 50 mM.
[b] Micromoles per minute per milligram of protein.
[c] ND, not determined.
[d] The enzyme activity for this substrate after 2 h of incubation was calculated with HPLC data.
[e] Micromoles of glucose produced per minute per milligram of protein.

animals such as sheep and goats, which are likely to encounter such cyanogenic plant species, this situation can depress animal performance and, at worst, lead to death (2, 14). Ruminal cellodextrinases may play a role in the sensitivity of ruminant animals towards plant-derived cyanogenic glycosides. Although their primary function is the hydrolysis of cellodextrins from plant-based cellulose, inopportune cross-reactivity with potentially dangerous carbohydrates is a realistic problem.

Two cyanogenic glycosides, amygdalin and prunasin, were tested as substrates for CdxA. In the case of prunasin, activity was highly significant at 71% of the rate of PNPG hydrolysis. Although CdxA had very low activity on gentiobiose, it was able to cleave amygdalin almost five times faster; this was possibly due to a lower binding constant. Amygdalin was hydrolyzed in a two-step process. A glucose molecule was removed initially from the nonreducing end to produce prunasin. The prunasin was then hydrolyzed, to yield glucose and mandelonitrile. Since mandelonitrile could not be detected with the HPLC column and solvent system available for this study, further substrate processing by the enzyme could not be monitored.

This work has demonstrated that one cellodextrinase, CdxA from *P. ruminicola*, can catalyze the first step in cyanide release from prunasin and amygdalin. The high cyanogenic activity of mixed populations of ruminal bacteria (19, 20) suggests that this is not an isolated case and that other enzymes with activity towards these cyanogenic compounds are present in the rumen. Preliminary qualitative studies indicate that CdxA is also capable of releasing free cyanide from both prunasin and amygdalin (data not shown). The extent and efficiency of this hydrolysis, however, are not yet known. Further work using an improved König cyanide assay (in progress), as well as an antibody screen to assess how widespread this enzymatic activity is among various ruminal bacterial strains and mixed populations, will help to identify specific herds as well as individual animals which are at a higher risk of being poisoned than normal.

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