JOURNAL OF BACTERIOLOGY, Dec. 1995, p. 7178–7185 Vol. 177, No. 24
0021-9193/95/$04.00+0

Purification of the Copper Response Extracellular Proteins Secreted by the Copper-Resistant Methanogen *Methanobacterium bryantii* BKYH and Cloning, Sequencing, and Transcription of the Gene Encoding These Proteins

BYOUNG-KWAN KIM,[1] TODD D. PIHL,[2] JOHN N. REEVE,[2] AND LACY DANIELS[1]*

Department of Microbiology and Center for Biocatalysis and Bioprocessing, University of Iowa, Iowa City, Iowa 52242,[1] and Department of Microbiology, The Ohio State University, Columbus, Ohio 43210[2]

Received 8 May 1995/Accepted 16 October 1995

When the copper-resistant methanogen *Methanobacterium bryantii* BKYH was exposed to 1 mM Cu(II), it secreted approximately fourfold increased levels of three proteins, copper response extracellular (CRX) proteins. The members of the CRX protein trio had apparent molecular masses of 40.8, 42.3, and 42.9 kDa and were purified together from the culture supernatant and separated from each other by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The N-terminal amino acid sequences of the three proteins were essentially identical, and antibodies raised against one of the trio reacted with all three proteins and with three other intracellular proteins with slightly higher molecular weights. The N-terminal amino acid sequence of one of these larger proteins was different from that of the secreted CRX proteins. The gene *crx*, which encodes the CRX proteins, was cloned and sequenced, and *crx* transcription was characterized. The *crx* sequence predicts that the encoded polypeptide is synthesized as a precursor with an N-terminal leader peptide, containing 28 amino acid residues, that is removed during the extracellular secretion of the CRX proteins. Transcription was initiated 274 bp upstream from the *crx* gene, producing an ~1.4-kb monocistronic transcript that was present in *M. bryantii* BKYH cells under all growth conditions but that increased approximately fourfold in vivo in response to Cu addition. The CRX proteins appear to be glycosylated, since they react with concanavalin A and neuraminidase, and to be the products of one gene that have different levels of posttranslational glycosylation. This is supported by very similar chromatographic and electrophoretic properties, identical N-terminal amino acid sequences, immunological cross-reactivities, and the detection of only one *crx*-related sequence by Southern blotting. Western blots (immunoblots) showed no evidence for CRX proteins in cell lysates of several other *Methanobacterium* strains.

Copper(II) is an essential trace element, but like many other heavy metals, above certain concentrations it is toxic to cells. Specific transport and enzyme-metal incorporation mechanisms allow the selective use of other metals in the presence of Cu, but some microorganisms further reduce the active copper concentration by detoxification mechanisms such as extracellular precipitation, binding and sequestration in complexes, and energy-dependent copper efflux (5–7, 11, 12, 14–16, 19, 20, 22, 34, 35, 37). These mechanisms have been well studied for aerobes and facultative aerobes (especially *Escherichia coli* and *Pseudomonas* spp.) but not for anaerobes, including the methanogens.

Understanding the effects of heavy metals on methanogens is important since methanogens play an essential role in anaerobic digesters and high concentrations of heavy metals are frequently present in wastewaters (1, 2, 24, 31). Some agricultural and industrial wastes have very high copper contents, and developing and manipulating anaerobic copper tolerance could allow more effective treatment of these wastes (17, 38) and could be of value in formulating a rational process to accelerate the recovery of waste treatment facilities from excessive copper exposure. Heavy-metal resistance might also provide a genetic tool for studying methanogens, which are inherently resistant to most antibiotics commonly used for selections in genetic experiments (8).

Recently we isolated a copper-resistant methanogen, *Methanobacterium bryantii* BKYH, from a copper mining area in the Upper Peninsula of Michigan (26). This isolate grows in media containing copper concentrations up to 30 times higher than the MICs known for other methanogens (2, 24, 26). We have investigated the response of this methanogen to copper and describe in this paper the purification and characterization of the copper response extracellular (CRX) proteins that are secreted at higher levels by this methanogen in response to copper exposure. The *crx* gene that encodes the CRX proteins has also been cloned and sequenced, and we have shown that the increase of copper concentration in the medium also increases the intracellular level of the *crx* transcript, indicating the presence of a copper-responsive archaeal promoter.

MATERIALS AND METHODS

Culture conditions. The copper-resistant methanogen *M. bryantii* BKYH was maintained at 37°C on isolate medium that contained 1 mM $CuCl_2$ as described by Kim et al. (26). Fermentor cultures were grown in the same medium, but cysteine was added by syringe to the medium during in situ sterilization. The fermentors used had working volumes of 12.5 and 20 liters, which were agitated at impeller settings of 400 and 250 rpm, respectively. Cultures were grown at 37°C and supplied with H_2-CO_2 (80:20, vol/vol) at a flow rate of 1 liter/min. *M. bryantii* MoH (from R. S. Wolfe, University of Illinois) and *Methanobacterium formicicum* (DSM 1312; Deutsche Sammlung von Mikroorganismen, Braunschweig, Germany) were cultivated at 37°C on isolate medium. *Methanobacterium thermoautotrophicum* Marburg was cultivated at 63°C as previously described (27) except that cysteine replaced sulfide.

* Corresponding author. Mailing address: Department of Microbiology, University of Iowa, Iowa City, IA 52242. Phone: (319) 335-4909. Fax: (319) 335-4901. Electronic mail address: lacy-daniels@uiowa.edu.

Copper challenge experiment. Serum-bottle or fermentor grown cultures were inoculated without copper addition. Anaerobic $CuCl_2$ was added when the culture optical density at 600 nm (OD_{600}) reached ~0.3, and cultivation was continued. For sample processing, EDTA was added to serum-bottle grown cultures to give a final concentration of 10 mM, and the samples were kept on ice for 10 min. Cells were removed by centrifugation (17,000 × *g*, 15 min), and the supernatant was passed through a 0.22-μm-pore-size filter to prepare cell-free culture supernatants. Proteins from 100-ml aliquots of cell-free culture supernatants were precipitated by the addition of cold trichloroacetic acid (final concentration, 10%) and collected, after incubation on ice for 1 h, by centrifugation (27,000 × *g*, 30 min). The resulting pellet was dissolved in 1 ml of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8) (3) and stored at −70°C. Fermentor cell-free culture supernatants were processed similarly, but a membrane concentrator (Centriprep-10; Amicon, Beverly, Mass.) with a 10-kDa nominal molecular-mass cutoff was used instead of trichloroacetic acid precipitation to concentrate the dissolved proteins.

Protein purification from culture supernatant. When fermentor culture turbidity reached an OD_{600} of ~0.3, anaerobic $CuCl_2$ was added to give a final concentration of 1 mM and cultivation was continued. At an OD_{600} of ~0.7, growth was stopped by chilling the cultures to ~15°C while H_2-CO_2 was still fed into the fermentor. Phenylmethylsulfonyl fluoride (PMSF) and EDTA were added to give final concentrations of 10 μM and 5 mM, respectively. Cells were harvested with a Pellicon cassette with Durapore polyvinylidene difluoride membranes (Millipore, Bedford, Mass.) with a 0.45-μm pore size and 0.38 m^2 of filter area. The filtrate was collected in a glass reservoir held in an ice bucket, and the retentate was returned to the fermentor. The final retentate (~0.75 liter) was centrifuged (6,000 × *g*, 30 min), and the cell pellet obtained was stored at −70°C. The filtrate (~11 liters) was further filtered through 0.22-μm-pore-size filters (Nalge, Rochester, N.Y.) at 4°C to prepare cell-free culture supernatant. Proteins in these filtrates were concentrated to a final volume of 300 to 400 ml by using a Prep/Scale TFF-6 cartridge (Millipore) with a 0.56-m^2 filter area and a 5-kDa nominal molecular-mass cutoff.

Proteins that precipitated at 40% ammonium sulfate saturation (4°C) were removed by centrifugation (27,000 × *g*, 30 min), and then proteins that precipitated at 80% ammonium sulfate saturation were collected by centrifugation, resuspended in TE buffer (with 10 μM PMSF), and dialyzed overnight at 4°C with membrane tubing (6- to 8-kDa nominal molecular-mass cutoff) in 220 volumes of buffer A (20 mM Tris-HCl–7 mM 2-mercaptoethanol–1 mM EDTA–5 μM PMSF–5% [vol/vol] glycerol, pH 8.5). The dialyzed material was applied to a fast-performance liquid chromatography anion exchange column (15 ml; Q-Sepharose; Pharmacia, Piscataway, N.J.) equilibrated with buffer A. Buffer B (2 M NaCl in buffer A) was used with buffer A for elution and regeneration. Fractions from the Q-Sepharose column were examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and fractions eluting at NaCl concentrations between 0.1 and 0.16 M were pooled and dialyzed in buffer C (20 mM MES [morpholineethanesulfonic acid]–7 mM 2-mercaptoethanol–1 mM EDTA–5 μM PMSF–5% [vol/vol] glycerol, pH 6.1). The dialyzed material was applied to a fast-performance liquid chromatography cation exchange column (15 ml; S-Sepharose; Pharmacia) equilibrated with buffer C and was eluted with a gradient of buffer C and buffer D (2 M NaCl in buffer C). Fractions from the S-Sepharose column were examined by SDS-PAGE, and fractions eluting at NaCl concentrations between 0.15 and 0.26 M were pooled. Glycerol was added to give a 30% (vol/vol) concentration, and the material was stored at −20°C.

Production of polyclonal antisera. The three polypeptides in 75 μg of the purified protein solution were separated by electrophoresis on two SDS–10% polyacrylamide gels. The fastest migrating of the three bands was cut from the gels with a razor blade on a light box following Coomassie brilliant blue staining. These gel pieces were destained with destaining solution, rinsed with saline several times, homogenized, and transferred into a 10-ml glass syringe connected to a second syringe by a double-ended Luer-Lock connector. For the initial immunization, 3 ml of Freund's complete adjuvant was homogenized with the gel by forcing the mixture from one syringe to the other until an emulsion formed. For booster injections, the gel was homogenized with 3 ml of sterile saline by the same method. For the initial immunization, the antigen mixture was injected at several sites subcutaneously on the back of a 4-kg New Zealand White male rabbit. Booster immunizations at multiple intramuscular sites were given twice at 2-week intervals. Blood samples, taken before immunization and 10 days after every injection, were treated as described previously (13). Sera were frozen in 100-μl amounts.

Cell lysate preparation. Cell pellets were resuspended in 1/10 of the original culture volume in TE buffer that contained 40 μM PMSF, and cells were broken by a French press (2 cycles at 18,000 lb/in^2). The lysates were stored at −70°C until use.

Purification of the precursor proteins. The method used to purify the mature CRX proteins from the culture supernatants was also used to purify the precursor proteins from cell lysates. However, a Western blot (immunoblot) procedure was needed, in addition to Coomassie staining, to identify those proteins in the fractions analyzed by SDS-PAGE. Antibodies raised against a mature CRX protein purified from the culture supernatant were used as the primary antibodies in the Western blots.

Gel electrophoresis and Western blotting. SDS-PAGE was used to analyze samples. Protein standards (no. 161-0304; Bio-Rad, Richmond, Calif.) were used to estimate molecular weights. Polypeptides in gels were stained with Coomassie brilliant blue R-250, and then gels were air dried between gel-drying films (Promega, Madison, Wis.). Band intensities were digitized with a model QCS 1260 flatbed scanner (Imapro; Ottawa, Ontario, Canada) with Adobe Photoshop software, and the images obtained were analyzed on a Silicon Graphics computer at the University of Iowa Image Analysis Facility. A Mini-Protean II gel electrophoresis system (60 by 100 by 1 mm; Bio-Rad) was used for one- or two-dimensional SDS-PAGE. Bio-lyte 5/7 and 3/10 ampholytes (Bio-Rad) were used to prepare 4% (wt/vol) polyacrylamide urea isoelectric focusing gels as described by the manufacturer (M1652960 RevB; Bio-Rad). A Protean II xi electrophoresis system (Bio-Rad) was used with SDS-10% polyacrylamide gels (200 by 200 by 1.5 mm) to prepare samples for N-terminal amino acid sequencing and immunization.

For Western blot analyses, after SDS-PAGE proteins were transferred by electrophoresis to polyvinylidene difluoride membranes in 10 mM CAPS (3-cyclohexyl-amino-1-propanesulfonic acid) buffer, which contained 10% (vol/vol) methanol at pH 11. Tris-HCl (10 mM, pH 7.4) buffer that contained 0.15 M NaCl and 5% skim milk (Difco, Detroit, Mich.) was used as the blocking buffer, and this buffer with 0.5% skim milk was used as the washing buffer. Antiserum raised against a protein purified from the culture supernatant was diluted 1,000-fold in the washing buffer for use as the primary antibody, and the secondary antibody (goat anti-rabbit immunoglobulin G antibody conjugated with alkaline phosphatase), was similarly diluted 1,000- to 2,000-fold. An ImmunoPure NBT/BCIP kit (Pierce, Rockford, Ill.) was used for color development.

N-terminal amino acid sequencing. Proteins were transferred by electrophoresis from an SDS-polyacrylamide gel to a polyvinylidene difluoride membrane in CAPS buffer and were stained with Coomassie brilliant blue. Bands were cut from the membrane and sequenced by an Applied Biosystems 475A protein sequencer in the University of Iowa Protein Structure Facility.

Glycoprotein identification and deglycosylation. A LectinLink kit (Genzyme, Cambridge, Mass.) was used to assay for protein glycosylation. Proteins were separated by SDS-PAGE and transferred by electrophoresis onto a nitrocellulose membrane in Tris-glycine buffer (25 mM Tris–200 mM glycine–20% [vol/vol] methanol, pH 8.8). The glycoprotein standards provided with the kit were used as controls. After the fixing and blocking steps, the membrane was cut into five strips, and each strip was incubated for 1 h in a biotinylated lectin solution, either concanavalin A, *Ricinus communis* agglutinin, *Datura stramonium* agglutinin, *Sambucus nigra* agglutinin, or wheat germ agglutinin. Each strip was then washed and incubated with an avidin-alkaline phosphatase conjugate for 1 h and then was subjected to color development.

Proteins were incubated at 37°C for ~12 h with one or more of four deglycosylases (Genzyme), and SDS-PAGE was used to examine the reaction mixtures. For reactions with endoglycosidase F_2 (endo F_2) (EC 3.2.1.96), the protein samples were mixed with SDS to give a 1% (wt/vol) final concentration and then incubated in a boiling water bath for 5 min and cooled. The reaction mixtures were then prepared following the manufacturer's protocol, with 6 μl of the SDS-protein solution and 3 μl of enzyme (200 mU/ml) diluted in buffer to a 30-μl final volume. Identical mixtures prepared without enzyme provided negative controls.

The substrate solution for digestions with peptide-N^4-(*N*-acetyl-β-glucosaminyl)-asparagine amidase (*N*-glycanase) (EC 3.5.1.52) was prepared by mixing 20 μl of purified protein solution with 2.5 μl of reaction buffer (500 mM Tris-HCl–2.5% [wt/vol] SDS–50 mM 2-mercaptoethanol, pH 7.5) and incubating this mixture in a boiling water bath for 5 min. Triton X-100 was added to a 4% (vol/vol) final concentration, and 0.4 U of *N*-glycanase was added before the volume was adjusted to 40 μl with deionized water.

For digestion with the endo-α-*N*-acetylgalactosaminidase (*O*-glycanase) (EC 3.2.1.97) or the acylneuraminyl hydrolase (neuraminidase) (EC 3.2.1.18), the storage buffer of the purified protein sample was exchanged with 20 mM bis-Tris buffer (adjusted to pH 6.6 with acetic acid) with a Centricon-10 device, and the volume was reduced to 1/30 of the original volume. This solution was mixed with SDS (final concentration, 0.1% [wt/vol]) and incubated in boiling water for 5 min, and Nonidet P-40 (Sigma Chemical Co., St. Louis, Mo.) was then added to give a 0.75% (vol/vol) final concentration. This substrate solution was mixed well with neuraminidase, calcium sulfate was added to 10 mM, and the mixture was incubated for 4 h at 37°C. *O*-Glycanase was added to half of this reaction mixture, and both reaction mixtures were then incubated for 14 h at 37°C. SDS-PAGE loading buffer was added to stop the reaction.

Protein assay. Protein was measured by the Bradford method (9) with the dye reagent purchased from Bio-Rad.

Cloning and DNA sequencing. Oligonucleotide probes were end labeled with digoxigenin-ddUTP (Boehringer-Mannheim, Indianapolis, Ind.) and hybridized to Southern blots of restriction enzyme digests of *M. bryantii* BKYH genomic DNA that was isolated and digested as described previously (3). On the basis of the results of these Southern hybridizations, *Eco*RI-generated restriction fragments of *M. bryantii* BKYH genomic DNA, between 3 and 4 kb in length, were isolated from gel slices digested with agarase, following the separation of the fragments by agarose gel electrophoresis, and were cloned into λgt11 (3). The recombinant phages obtained were plated on *E. coli* Y1090r$^-$ and screened by plaque hybridization (3) for phages that contained DNA sequences complemen-

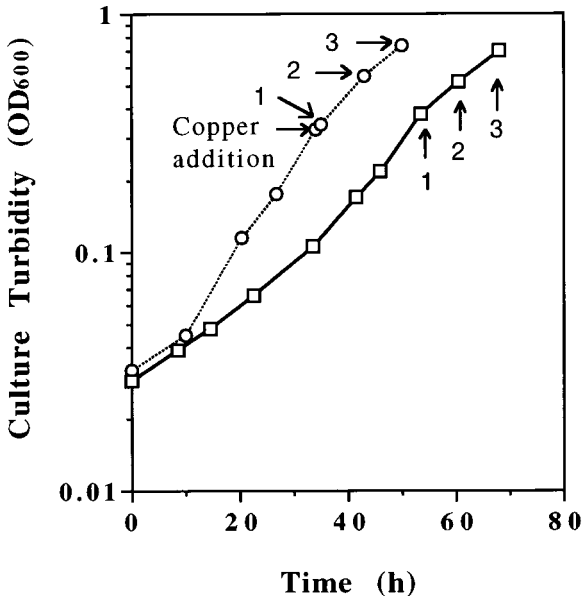

FIG. 1. Copper challenge experiment with fermentor-grown cultures. When cultures were at an OD_{600} of 0.3, $CuCl_2$ was added (○) or not added (□) to give a final concentration of 1 mM, and cultivation was continued. The sample numbers and arrows indicate when protein samples were collected from the cell-free culture supernatant.

tary to the oligonucleotide probes. Two positive clones were identified, and both contained the same-sized, ~3.7-kb *Eco*RI fragment, which was then subcloned into pBluescript II SK^- (Stratagene, La Jolla, Calif.), generating pBKY101 and pBKY102. DNA sequences were obtained from the plasmids at the University of Iowa DNA Facility with an Applied Biosystems model 373A sequencer with primers generated by an Applied Biosystems model 394 oligonucleotide synthesizer. The sequences obtained were analyzed with the Gene Jockey (Biosoft, Cambridge, United Kingdom) and Genetics Computer Group (University of Wisconsin Biotech Center, Madison, Wis.) programs and were compared with all database sequences through the National Center for Biotechnology Information.

Transcriptional analysis. Total RNA from *M. bryantii* BKYH was isolated as described before (32), except that the cells were ruptured by passage through a French pressure cell at 15,000 lb/in^2. Primer extension reactions were conducted as described by Montzka and Seitz (30). Northern (RNA) blot analyses used ^{32}P-end-labeled probes, and the blots were quantified directly with a Packard Instant Imager 2024 (23, 32).

Nucleotide sequence accession number. The sequence of the *crx* gene has been deposited in GenBank under accession no. U40213.

RESULTS

Copper challenge experiments. SDS-PAGE examination of the proteins in the supernatants taken at different times from bottle cultures grown with no addition of copper or at copper concentrations of 0.5 or 1.0 mM revealed that the amounts of three proteins with apparent molecular masses of 40 to 43 kDa increased more than those of other proteins in the cultures grown with copper (data not shown). This was examined systematically with fermentor-grown cultures.

Although there was some culture-to-culture variation in growth rates, overall growth patterns for cultures grown with and without copper addition were very similar. Samples taken at approximately the same OD_{600} during exponential growth (Fig. 1) were analyzed by SDS-PAGE (Fig. 2). The proteins of interest, first observed in bottle-grown cultures, were also present at high levels in fermentor supernatants in copper-exposed cultures, compared with those not exposed to copper. Image analyses confirmed that the region from 40 to 43 kDa contained three bands. Intensity changes of these three bands were calculated on the basis of the total intensity of each lane.

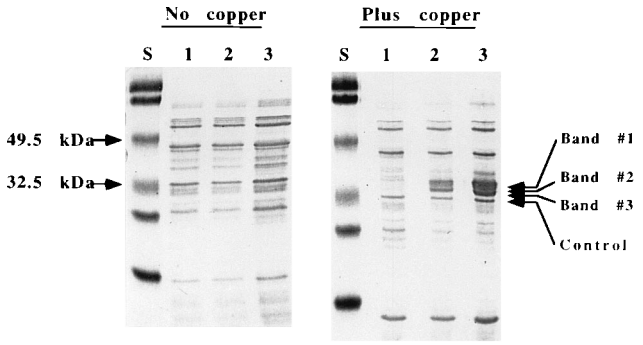

FIG. 2. SDS-PAGE of protein samples prepared from a copper challenge experiment. Samples of cell-free culture supernatants, taken from fermentor-grown cultures during the copper challenge experiment (Fig. 1), were examined by SDS-PAGE (12% polyacrylamide); quantities applied were based on an equal sample volume basis. Gels were stained with Coomassie brilliant blue. The lane numbers correspond to the sample numbers in Fig. 1. Prestained protein standards (S) from Bio-Rad were used as molecular mass markers. These gels were scanned and used for the image analysis shown in Fig. 3.

A band at ~32.5 kDa was selected as a control because this band showed up in both cases and did not increase preferentially after copper addition. As shown in Fig. 3, after 15 h of incubation in the presence of copper the relative intensities of the three bands of interest increased approximately fourfold whereas there was little change in the level of the control protein. In contrast, the extracellular levels of these proteins, referred to in this work as CRX proteins, declined slightly during the same 15-h period of incubation with no addition of copper.

Purification and properties of CRX proteins. The three CRX proteins behaved similarly in all purification steps and were purified from cell-free culture supernatant by monitoring their presence by SDS-PAGE. They precipitated at the same ammonium sulfate concentration and coeluted from Q-Sepha-

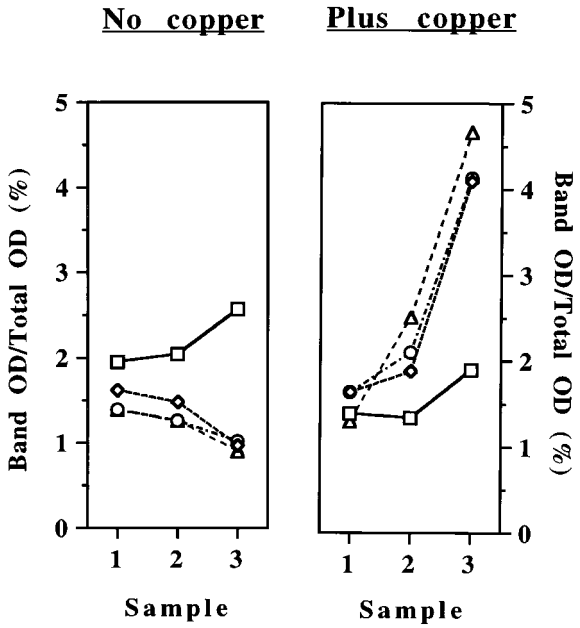

FIG. 3. Relative intensities of proteins of interest compared with total intensities of proteins in each lane of the gels shown in Fig. 2. □, control band; ◇, band 1; ○, band 2; △, band 3.

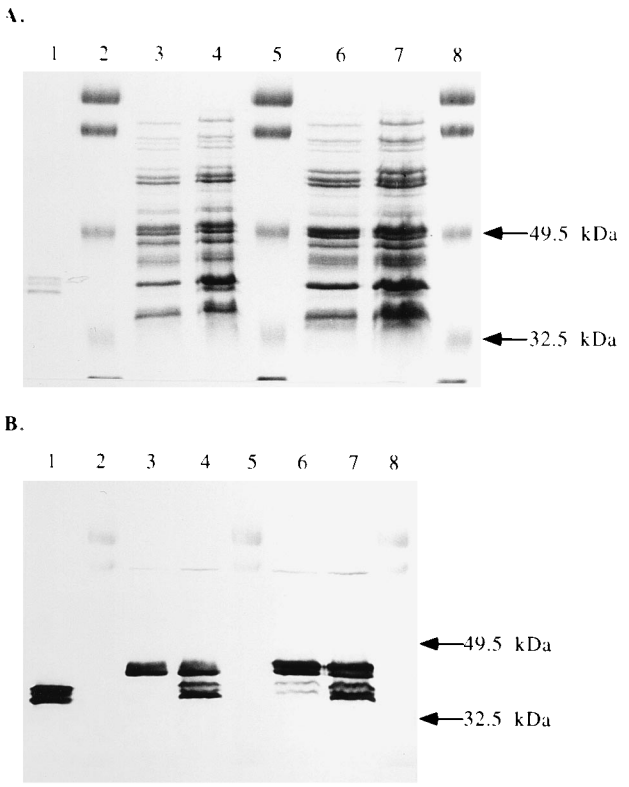

FIG. 4. Coomassie blue-stained gel (A) and Western blot (B) of cell lysates. Two SDS–10% polyacrylamide gels were run at the same time. Gel A was stained with Coomassie brilliant blue and gel B was analyzed by Western blotting. Lanes: 1, purified CRX proteins; 2, prestained protein standards; 3, lysate of cells grown without copper; 4, same as lane 3 plus CRX proteins; 5, prestained protein standards; 6, lysate of cells grown with copper; 7, same as lane 6 plus CRX proteins; 8, prestained protein standards. The lanes contained approximately equal amounts of protein.

rose and S-Sepharose. A two-dimensional gel electrophoretic analysis, in which the first dimension used an isoelectric focusing gel in a capillary column and the second dimension used an SDS-polyacrylamide slab gel, showed that all three proteins had the same apparent pI of 8.7. The final purified product of ~400 μg (6 ml) of CRX protein obtained from a 12.5-liter fermentor culture contained all three proteins, which had apparent molecular masses of 40.8, 42.3, and 42.9 kDa.

Rabbit antibodies were raised against the smallest of the three proteins. Preimmune rabbit serum did not contain antibodies that bound to the CRX proteins. The anti-40.8-kDa CRX protein serum did, however, contain antibodies that bound to all three CRX proteins and also to three intracellular proteins whose electrophoretic mobilities corresponded to molecular masses of 45 to 48 kDa (Fig. 4). These proteins were purified from lysates of *M. bryantii* BKYH cells grown in the presence of copper.

N-terminal amino acid sequences. The N-terminal amino acid sequences determined for the 40.8-, 42.3-, and 42.9-kDa CRX proteins purified from culture supernatants were TASA DNKVRVGYLP(D/S)TGD(F/S)LYFIA(G/K), TASADNKV RVGYLPDTGD(F/S)LYFIAK, and TASADNKVRVGYLP STGD(F/S)LYFIAK, respectively. All three sequences appear to be the same, with ambiguities at positions 15, 19, and 25 always involving common amino acid residues. These sequences, and all variations arising from the ambiguities, were compared with the peptide sequence databases at the National

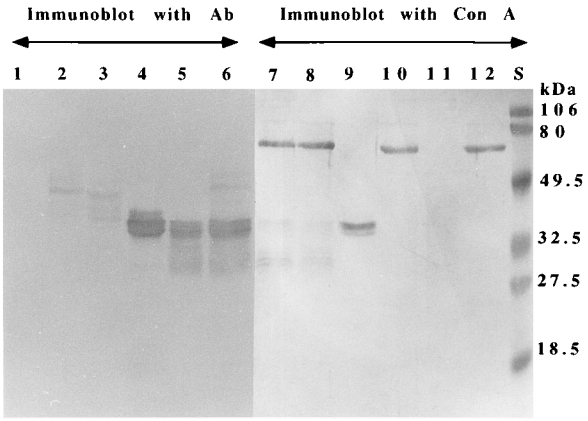

FIG. 5. Western blots demonstrating deglycosylation of the CRX proteins. Purified CRX proteins were incubated with neuraminidase (NA) or *O*-glycanase (OG), and SDS-PAGE (12% polyacrylamide) was run with prestained protein standards (S). Proteins were then electrophoretically transferred onto a nitrocellulose membrane, and the membrane was cut into two halves. One half (lanes 1 through 6) was incubated with the anti-40.8-kDa CRX protein antiserum, and the other half (lanes 7 through S) was incubated with concanavalin A (Con A). Lanes 1 and 12, NA only; lanes 2 and 11, OG only; lanes 3 and 10, NA + OG; lanes 4 and 9, CRX proteins only; lanes 5 and 8, CRX proteins + NA; lanes 6 and 7, CRX proteins + NA + OG.

Center for Biotechnology Information, but no significant similarities between the CRX protein sequences and database proteins were detected.

N-terminal amino acid sequencing was carried out for the largest protein of the three intracellular proteins that reacted with the anti-40.8-kDa antibodies. The sequence obtained, VNIDKKIVII, was different from the N-terminal amino acid sequences of the three supernatant CRX proteins, suggesting that the larger proteins could be intracellular precursors of the proteins purified from the supernatants.

Identification of posttranslational modifications to the CRX proteins. The three CRX proteins bound to concanavalin A (Fig. 5), a lectin which binds to terminal α-D-mannopyranosyl or α-D-glucopyranosyl residues (36), indicating that these are glycoproteins which have one or both of these sugar residues. The other lectins tested did not bind to the CRX proteins (data

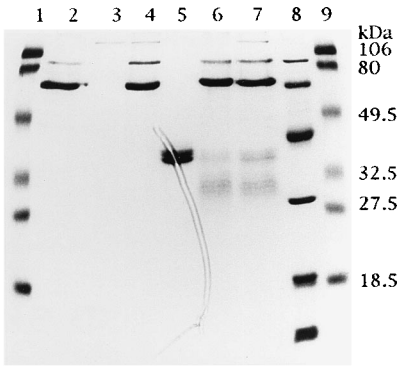

FIG. 6. Coomassie brilliant blue-stained gel showing deglycosylation of the CRX proteins. Purified CRX proteins were incubated with neuraminidase (NA) or *O*-glycanase (OG), and following separation of the products by SDS-PAGE (12% polyacrylamide) the gel was stained with Coomassie brilliant blue. Lanes 1 and 9, prestained protein standards; lane 2, NA only; lane 3, OG only; lane 4, NA + OG; lane 5, CRX proteins only; lane 6, CRX proteins + NA; lane 7, CRX proteins + NA + OG; lane 8, unstained protein standards (97.4, 66.2, 45.0, 31.0, 21.5, and 14.4 kDa).

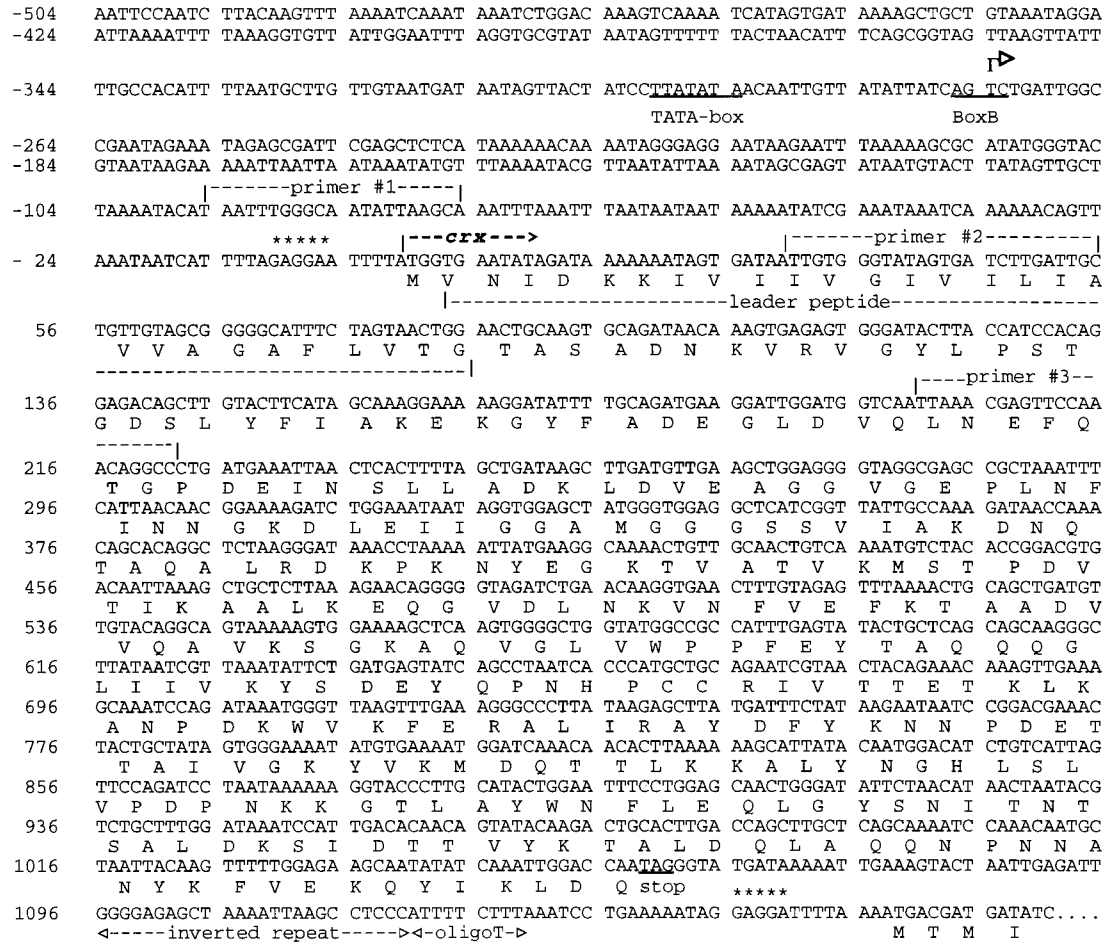

FIG. 7. DNA sequence determined for the *crx* gene and flanking regions. The A of the ATG initiation codon of the *crx* gene is designated as nucleotide position 0. The sequences complementary to the oligonucleotides used as the primers and probe to detect and characterize the *crx* transcript (Fig. 8 and 9) are designated primers 1, 2, and 3. The putative TATA-box and BoxB elements of the *crx* promoter are underlined, with the site of *crx* transcription initiation identified by the open arrow within the BoxB sequence (see Fig. 8). The putative ribosome-binding site is indicated by asterisks above the bases. Encoded amino acids are identified under the central nucleotide of their encoding codons. An inverted-repeat sequence in the intergenic region downstream from the *crx* gene, which could direct *crx* transcription termination at the following oligo(dT) sequence, is identified.

not shown), indicating the absence of a terminal galactose, *N*-acetylglucosamine, *N*-acetyllactosamine, or sialic acid residues attached to galactose in α-2,6 or α-2,3 linkages.

Digestion with neuraminidase reduced the molecular weights of the CRX proteins. The intensities of these proteins were reduced in samples exposed to neuraminidase (lanes 5, 6, 7, and 8 in Fig. 5 and lanes 6 and 7 in Fig. 6) compared with samples not treated with neuraminidase (lanes 4 and 9 in Fig. 5 and lane 5 in Fig. 6). On the basis of the electrophoretic mobilities of the standards in lane 8 of Fig. 6, CRX protein molecular masses were reduced from ~40 to 43 kDa to ~31.5 to 35 kDa by neuraminidase digestion. This further supports the argument that the CRX proteins are glycoproteins and also suggested that molecular mass differences among the three CRX proteins or their putative precursors might be caused by different levels of glycosylation of one gene product. The 97-kDa band visible in all samples that contained neuraminidase is neuraminidase (28), and the band of ~66 kDa that appeared in these samples on a Western blot with concanavalin A (Fig. 5) and on Coomassie blue-stained gels (Fig. 6) was identified as bovine serum albumin (BSA) by Genzyme (personal communication). BSA has no enzymatic activity and was used as an enzyme stabilizer.

By comparison of lanes 5 and 6 or 7 and 8 in Fig. 5 and lanes 6 and 7 in Fig. 6, it can be concluded that *O*-glycanase had no activity on these proteins, although the 160-kDa *O*-glycanase band was detected on a Coomassie blue-stained gel (Fig. 6). Western blots with both the preimmune and anti-40.8-kDa CRX protein sera revealed impurities in the *O*-glycanase, with molecular masses of 35 to 50 kDa (lane 2 in Fig. 5), that bound antibodies in both of these antisera. These impurities were not degradation products of *O*-glycanase, because the 160-kDa, full-length *O*-glycanase did not give a positive signal on these Western blots.

Cloning and sequencing of the *crx* gene. A mixture of oligonucleotides was used as the probe to identify and clone the *crx* gene. This probe was synthesized to contain the sequence(s) 5′-(A/G)TCNGGNAG(A/G)TANCCNAC(T/C)CTNAC(T/C)TT(A/G)TT(A/G)TC-3′, which is based on the amino acid sequence DNKVRVGYLPD determined directly for positions 5 through 15 of the three purified CRX proteins. Probe molecules hybridized strongly to only one 3.7-kb restriction fragment in an *Eco*RI digest of *M. bryantii* BKYH genomic DNA. The *Eco*RI-generated restriction fragments, between 3 and 4 kb in length, were therefore isolated and cloned into λgt11. Two positive clones were identified, and both were found to

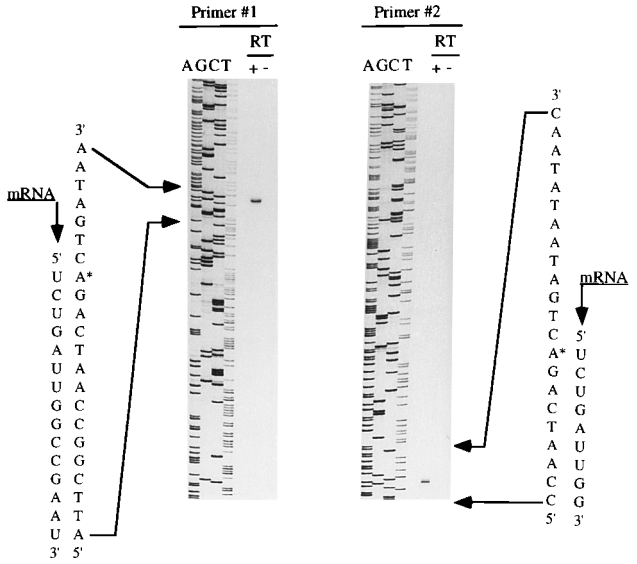

FIG. 8. Identification of the site of *crx* transcription initiation by primer extension analyses. Primers 1 and 2 were oligonucleotides synthesized with sequences complementary to the sequences identified as primers 1 and 2 in Fig. 7. Primer extension reactions were carried out with (+) and without (−) avian myeloblastosis virus reverse transcriptase (RT). The DNA sequencing ladders shown were obtained for the intergenic region upstream of the *crx* gene by using the same oligonucleotides as sequencing primers. The same site of *crx* transcription initiation, indicated by the asterisks, was identified independently with both primers.

contain the same ~3.7-kb *Eco*RI fragment, which was then subcloned into pBluescript to form pBKY101 and pBKY102. Further restriction mapping and Southern blot hybridizations revealed that the probe molecules hybridized to a 1.7-kb *Eco*RI-*Eco*RV fragment contained within the 3.7-kb *Eco*RI fragment, and this was therefore subcloned and sequenced.

Only one complete open reading frame was present in the DNA sequence obtained (Fig. 7). The encoded gene product (calculated molecular mass, 38.6 kDa) has the same N-terminal amino acid sequence as that determined directly for the largest of the three putative precursor forms of CRX protein, except that the purified gene product lacked an N-terminal methionyl residue. Codons 34 through 44 of this open reading frame encoded the amino acid sequence that was determined for residues 5 through 15 of the secreted CRX proteins and that was used to design the oligonucleotide probes. The *crx*-encoded amino acid sequence is not obviously related to sequences in the databases of known proteins; the two most similar proteins showed similarities of ~43 and ~18% identity, which, when analyzed according to the guidelines of Doolittle (18), suggested that there is no significant relationship.

Immediately upstream from the ATG translation-initiating codon of the *crx* gene is the sequence 5′-GAGGA, which is consistent with a methanogen ribosome-binding site. Downstream from the TAG translation-terminating codon is an inverted-repeat sequence followed by an oligo(dT) sequence, which is consistent with a methanogen transcription terminator (33).

Transcriptional analysis. To identify and characterize the *crx* transcript, three oligonucleotides, with sequences complementary to the DNA sequences identified as primers 1, 2, and 3 in Fig. 7, were synthesized. Two of these (primers 1 and 2) were used to identify the site of *crx* transcription initiation. Following the hybridization of primers to total RNA preparations, the extension products generated from both primers (Fig. 8) demonstrated that *crx* transcription was initiated 274 bp upstream from the ATG translation-initiating codon, at the T residue in a 5′-AGTC sequence (identified in Fig. 7). This sequence conforms to the BoxB motif established for transcription initiation sites in members of the domain *Archaea* and is located appropriately 20 bp downstream from the sequence 5′-TTATATA that conforms to the TATA-box motif of a methanogen promoter (33). Northern blots (Fig. 9) with oligonucleotide 3 as the probe demonstrated that the major *crx* transcript was ~1.4 kb in length, consistent with a monocistronic transcript initiated at nucleotide −274 and terminated, following the oligo(dT) sequence, at nucleotide ~1130 (Fig. 7). Much weaker hybridization signals suggested the presence of much smaller amounts of longer *crx*-containing transcripts that have not been further investigated.

To determine if *crx* transcription responded to Cu addition, RNA preparations were isolated from *M. bryantii* BKYH cells grown in a fermentor before and after Cu addition. As shown

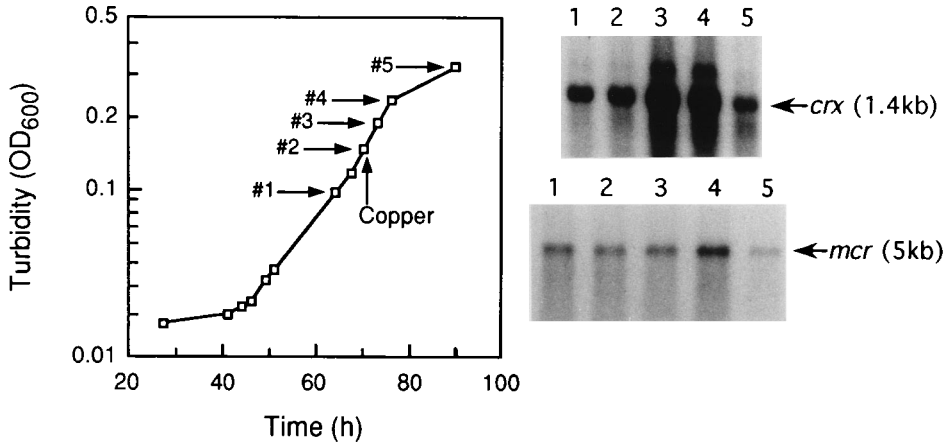

FIG. 9. Growth of *M. bryantii* BKYH in the presence of $CuCl_2$ (left panel) and Northern blots of RNA preparations before and after Cu addition (right panels). $CuCl_2$ was added (final concentration, 1 mM) immediately after sample 2 was removed. RNA preparations were isolated from cells taken from the culture at time points 1 through 5. To detect the *crx* message, primer 3 (Fig. 7) was used as the probe. To detect the *mcr* transcript, a mixture of oligonucleotides with the sequence(s) GA(G/A)ACCAT(C/T)TG(G/C/T)GTNCCNCG(A/G)TC was used as the probe. The tracks identified above the two Northern blots as 1 through 5 each contained 10 μg of total RNA isolated from the cells sampled from the culture at the corresponding time points indicated in the left-hand panel.

in Fig. 9, adding 1 mM $CuCl_2$ (immediately after time point 2) had no observable effects on the growth of the culture but did result in increased levels of the *crx* transcript. Northern blots generated with aliquots of total RNA isolated from cells taken from the cultures at different times before and after Cu addition were probed with both oligonucleotide 3, which was specific for the *crx* transcript (Fig. 7), and an oligonucleotide used previously to identify and quantitate transcripts of a very highly conserved region within the *mcr* operon that encodes methyl coenzyme-M methyl reductase I (32). When β-emissions from the Northern blots were quantitated, the amount of the *crx* transcript per 10 μg of total RNA was found to increase approximately fourfold following $CuCl_2$ addition whereas there was little change in the amount of the *mcr* transcript. This increased abundance of the *crx* transcript was maintained in *M. bryantii* BKYH cells grown in the presence of Cu until the culture reached the stationary growth phase, and then the level of transcript decreased.

Novelty of CRX proteins in *M. bryantii* BKYH. Cell lysates of close relatives of *M. bryantii* BKYH, namely, *M. bryantii* MoH and *M. formicicum*, and of a more distant relative, *M. thermoautotrophicum* Marburg, gave negative results when probed by Western blot with preimmune serum or anti-CRX protein serum (26). Negative results of Southern blots also indicated that the *crx* gene was not conserved in the genomes of *M. thermoautotrophicum* Marburg and ΔH, *M. formicicum*, or *Methanobacterium thermoformicicum*.

DISCUSSION

The presence of copper results in the coordinated approximately fourfold increase in the extracellular levels of three CRX proteins that appear to be all coded by the same gene (*crx*) on the basis of their identical N-terminal amino acid sequences, conserved epitopes, very similar electrophoretic and chromatographic properties, and Southern blot analyses that demonstrate the presence of only one *crx*-related sequence in the *M. bryantii* BKYH genome. This *crx* gene contains 354 codons which encode precursor and mature forms of the CRX polypeptide that have calculated molecular masses of 38.6 and 35.8 kDa, respectively. These values are 5 to 10 kDa less than the molecular masses estimated by SDS-PAGE for the three precursors and mature forms of CRX proteins, and the additional masses of these gene products apparently result from different levels of posttranslational glycosylation. Glycosylation may, in fact, be a common feature of extracellular proteins synthesized by methanogens, as this has also been reported for methanogen S-layer proteins and flagellins (4, 10, 21, 25). Ideally, if complete deglycosylation could be achieved the three CRX proteins would migrate as one band during SDS-PAGE after this treatment, but this was not the case. The *O*-glycanase and *N*-glycanase apparently could not cleave the bonds between the amino acid residues and glycosyl groups on the CRX proteins, and although neuraminidase had activity on the CRX proteins, this enzyme hydrolyzes only α-ketosidic linkages between terminal *N*-acetylneuraminic acid and sugar residues. Once it encounters a sugar residue other than an *N*-acetylneuraminic acid, digestion terminates and the rest of the carbohydrate moiety remains on the protein. This would explain the results shown in Fig. 6, in which the three CRX bands fade and several less-defined bands appear at ~32 kDa after neuraminidase exposure. Image analyses demonstrated that the same pixel number was present in the stained areas (data not shown), indicating that the fading was not due to overall protein loss. Similar data were reported for the *Methanococcus deltae* flagellins, for which hypoglycosylation (induced by bacitracin) resulted in the appearance of several lower-molecular-weight bands (4).

The function(s) of the CRX proteins is unknown; however, their syntheses are clearly regulated, at the level of either transcription initiation or *crx* transcript stability, by the presence of copper (Fig. 9). This, plus the existence of these proteins only in the copper-resistant methanogen *M. bryantii* BKYH, suggests that the CRX proteins are likely to be involved in copper resistance, although they could be synthesized as part of a more generic stress response. In addition to increasing the *crx* transcript levels, copper may also trigger the machinery that cuts off the leader sequence of the precursors and releases the proteins into the supernatant. One attractive model for CRX protein function would be as secreted and/or cell surface proteins that chelate copper and thereby effectively reduce the copper concentration in the medium immediately surrounding *M. bryantii* BKYH cells. This is consistent with the observations of Mittelman and Geesey (29), who demonstrated that a glycoprotein-exopolymer complex synthesized by a freshwater-sediment bacterium had copper-binding activity. Regardless of the CRX protein functions, if copper addition does, in fact, stimulate *crx* transcription initiation, this will provide a very attractive, experimentally tractable, and almost unique system to study promoter activation in an archaeon. The CRX protein maturation and secretion processes involve the removal of 28 N-terminal amino acid residues which form a polypeptide that has a length and hydrophobicity profile consistent with a leader peptide function in bacteria. This adds further support to previous genetic and physiological evidence from surface glycoproteins and flagellin (4, 10, 25, 33) that the basic features of protein secretion are conserved in *Bacteria* and in methanogenic *Archaea*.

ACKNOWLEDGMENTS

We thank Young Hwang and Kyoung Lee for discussions and assistance and Mike Feiss for help with sequence evaluations.

Research at The University of Iowa was supported by the U.S. Department of Agriculture through research grant 4132008 to the Biotechnology Byproducts Consortium, and research at The Ohio State University was supported by Department of Energy grant DE-FO2-87ER13731.

REFERENCES

1. **Ahring, B. K., and P. Westermann.** 1983. Toxicity of heavy metals to thermophilic anaerobic digestion. Eur. J. Appl. Microbiol. Biotechnol. **17:**365–370.
2. **Ahring, B. K., and P. Westermann.** 1985. Sensitivity of thermophilic methanogenic bacteria to heavy metals. Curr. Microbiol. **12:**273–276.
3. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl.** 1989. Short protocols in molecular biology, p. 1–387. *In* P. Wang-Iverson and S. G. Bonitz (ed.), Current protocols in molecular biology. John Wiley & Sons, New York.
4. **Bayley, D. P., M. L. Kalmokoff, and K. F. Jarrell.** 1993. Effect of bacitracin on flagellar assembly and presumed glycosylation of the flagellins of *Methanococcus deltae*. Arch. Microbiol. **160:**179–185.
5. **Bender, C. L., and D. A. Cooksey.** 1986. Indigenous plasmids in *Pseudomonas syringae* pv. *tomato*: conjugative transfer and role in copper resistance. J. Bacteriol. **165:**534–541.
6. **Bender, C. L., and D. A. Cooksey.** 1987. Molecular cloning of copper resistance genes from *Pseudomonas syringae* pv. *tomato*. J. Bacteriol. **169:**470–474.
7. **Bender, C. L., D. K. Malvick, K. E. Conway, S. George, and P. Pratt.** 1990. Characterization of pXV10A, a copper resistance plasmid in *Xanthomonas campestris* pv. *vesicatoria*. Appl. Environ. Microbiol. **56:**170–175.
8. **Böck, A., and O. Kandler.** 1985. Antibiotic sensitivity of archaebacteria, p. 525–544. *In* C. R. Woese and R. S. Wolfe (ed.), The bacteria. Academic Press, Orlando, Fla.
9. **Bradford, M. M.** 1977. A rapid and sensitive method for the quantitation of microgram quantities of protein using the principle of protein-dye binding. Anal. Biochem. **72:**248–254.
10. **Bröckl, G., M. Behr, S. Fabry, R. Hensel, H. Kaudewitz, E. Biendl, H. Konig.** 1991. Analysis and nucleotide sequence of the genes encoding the surface-layer glycoproteins of the hyperthermophilic methanogens *Methanothermus*

fervidus and *Methanothermus sociabilis*. Eur. J. Biochem. **199:**147–152.
11. **Brown, N. L., D. A. Rouch, and B. T. Lee.** 1992. Copper resistance determinants in bacteria. Plasmid **27:**41–51.
12. **Clarke, S. E., J. Stuart, and J. Sanders-Loehr.** 1987. Induction of siderophore activity in *Anabaena* spp. and its moderation of copper toxicity. Appl. Environ. Microbiol. **53:**917–922.
13. **Coligan, J. E.** 1991. Current protocols in immunology. John Wiley & Sons, New York.
14. **Cooksey, D. A.** 1987. Characterization of a copper resistance plasmid conserved in copper-resistant strains of *Pseudomonas syringae* pv. *tomato*. Appl. Environ. Microbiol. **53:**454–456.
15. **Cooksey, D. A.** 1993. Copper uptake and resistance in bacteria. Mol. Microbiol. **7:**1–5.
16. **Cooksey, D. A., H. R. Azad, J.-S. Cha, and C.-K. Lim.** 1990. Copper resistance gene homologs in pathogenic and saprophytic bacterial species from tomato. Appl. Environ. Microbiol. **56:**431–435.
17. **Davis, G. K.** 1974. High-level copper feeding of swine and poultry. Fed. Proc. **33:**1194–1196.
18. **Doolittle, R. F.** 1987. Of URFS and ORFS. University Science Books, Mill Valley, Calif.
19. **Erardi, F. X., M. L. Feilla, and J. O. Falkinham III.** 1987. Plasmid-encoded copper resistance and precipitation by *Mycobacterium scrofulaceum*. Appl. Environ. Microbiol. **53:**1951–1954.
20. **Gadd, G. M., and C. White.** 1989. Heavy metal and radionuclide accumulation and toxicity in fungi and yeasts, p. 19–38. *In* R. K. Poole and G. M. Gadd (ed.), Metal-microbe interactions. Academic Press, Oxford.
21. **Hartmann, E., and H. König.** 1989. Uridine and dolichyl diphosphate activated oligosaccharides are intermediates in the biosynthesis of the S-layer glycoprotein of *Methanothermus fervidus*. Arch. Microbiol. **151:**274–281.
22. **Harwood-Sears, V., and A. S. Gordon.** 1990. Copper-induced production of copper-binding supernatant proteins by the marine bacterium *Vibrio alginolyticus*. Appl. Environ. Microbiol. **56:**1327–1332.
23. **Hennigan, A. N., and J. N. Reeve.** 1994. mRNAs in the methanogenic archaeon *Methanococcus vannielii*: numbers, half-lives and processing. Mol. Microbiol. **11:**655–670.
24. **Hobson, P. N., and B. G. Shaw.** 1976. Inhibition of methane production by *Methanobacterium formicicum*. Water Res. **10:**849–852.
25. **Kalmokoff, M. L., T. M. Karnauchow, and K. F. Jarrell.** 1990. Conserved N-terminal sequences in the flagellins of archaebacteria. Biochem. Biophys. Res. Commun. **167:**154–160.
26. **Kim, B.-K., E. Conway de Macario, and L. Daniels.** Isolation and characterization of a copper-resistant methanogen from a copper mining soil sample. Submitted for publication.
27. **Kim, B.-K., and L. Daniels.** 1991. Unexpected error in gas chromatographic analysis of methane production by thermophilic bacteria. Appl. Environ. Microbiol. **57:**1866–1869.
28. **Kiyohara, T., T. Terao, K. Shioiri-Nakano, and T. Osawa.** 1974. Purification and properties of a neuraminidase from *Streptococcus* K 6646. Arch. Biochem. Biophys. **164:**575–582.
29. **Mittelman, M. W., and G. G. Geesey.** 1985. Copper-binding characteristics of exopolymers from a freshwater-sediment bacterium. Appl. Environ. Microbiol. **49:**846–851.
30. **Montzka, K. A., and J. A. Steitz.** 1988. Additional low-abundance human small nuclear ribonucleoproteins: U11, U12 etc. Proc. Natl. Acad. Sci. USA **85:**8885–8889.
31. **Parkin, G. F., R. E. Speece, C. H. J. Yang, and W. M. Kocher.** 1983. Response of methane fermentation systems to industrial toxicants. J. Water Pollut. Control Fed. **55:**44–53.
32. **Pihl, T. D., S. Sharma, and J. N. Reeve.** 1994. Growth phase-dependent transcription of the genes that encode the two methyl coenzyme M reductase isoenzymes and N^5-methyltetrahydromethanopterin: coenzyme M methyltransferase in *Methanobacterium thermoautotrophicum* ΔH. J. Bacteriol. **176:**6384–6391.
33. **Reeve, J. N.** 1992. Molecular biology of methanogens. Annu. Rev. Microbiol. **46:**165–191.
34. **Rouch, D., B. T. O. Lee, and J. Camakaris.** 1989. Genetic and molecular basis of copper resistance in *E. coli*, p. 439–446. *In* D. H. Hamer and D. R. Winge (ed.), Metal ion homeostasis: molecular biology and chemistry. Alan R. Liss, New York.
35. **Silver, S., and M. Walderhaug.** 1992. Gene regulation of plasmid- and chromosome-determined inorganic ion transport in bacteria. Microbiol. Rev. **56:**195–228.
36. **Singleton, P., and D. Sainsbury.** 1987. Dictionary of microbiology and molecular biology. John Wiley & Sons, New York.
37. **Tetaz, T. Z., and R. K. J. Luke.** 1983. Plasmid-controlled resistance to copper in *E. coli*. J. Bacteriol. **154:**1263–1268.
38. **Trevors, J. T., and C. M. Cotter.** 1990. Copper toxicity and uptake in microorganisms. J. Ind. Microbiol. **6:**77–84.