Isolation and Characterization of a Copper-Resistant Methanogen from a Copper-Mining Soil Sample

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A copper-resistant methanogen for which the CuSO₄ MICs were ~2- to 36-fold higher than those for other methanogens tested was isolated from a copper-mining area in the upper peninsula of Michigan. The rod-shaped methanogen used H₂-CO₂ or formate, but not acetate or methanol, as a growth substrate. Standing incubation with H₂-CO₂ medium resulted in a mat-like surface growth, dependent on the presence of hydrogen. The presence of 1 mM cupric salt resulted in longer filamentous and intertwined cells. Antigenic fingerprinting, 16S rRNA gene analysis, morphology, and substrate use suggest that the new isolate is a novel strain of *Methanobacterium bryantii* that is able to use formate.

Copper toxicity is useful to control agricultural pests and in paint to prevent ship biocorrosion. However, microorganisms can develop resistance mechanisms that allow growth at high copper concentrations, e.g., in farming areas in which copper biocides are used (9). With resistance, the application of copper must then be increased, causing increased pollution. As marine industries shift from alkyl-tin paints to copper-based paints for metal surfaces, copper levels may also increase in those environments.

Copper can also drain into anaerobic sewage digesters, inhibit methanogenic bacteria and other anaerobes in their ecosystem (2, 16), and shut down the methanogenesis process. This process can arise from the use of copper sulfate to control a variety of pests, from the release of industrial waste material containing copper, or from waste from animals being fed copper-supplemented feed (12, 43). If more were known about copper resistance in methanogens and other anaerobic bacteria, some wastes might be more effectively treated or a logical intervention strategy could be developed to readily return waste treatment facilities to acceptable levels of performance following exposure to copper.

There have been many studies of the mechanism of copper resistance in microorganisms and even on how to control the appearance of copper-resistant microorganisms (3, 8, 9, 15, 36, 38, 43), but most studies have been carried out with aerobes. Examination of the effects of copper and other heavy metals on anaerobes has been limited to descriptions of the responses of a few methanogens to metal exposure and the effect of several metals on overall methanogenesis in mixed cultures (1, 2, 16, 21, 33). Thus, we have undertaken a study of copper resistance mechanisms in methanogenic bacteria, since they are of importance in both anaerobic digestion and in metal biocorrosion (11), and in the hope we can learn more about heavy metal toxicity and resistance mechanisms in general. We report in this paper the isolation of the first copper-resistant methanogen, from a copper-mining area, and describe its properties. In another paper, we reported the purification of copper-re-

sponse proteins from this methanogen and the cloning, sequencing, and transcription of the gene coding for those proteins (20).

MATERIALS AND METHODS

Cultivation of known methanogens. *Methanobacterium formicicum* (DSM 1535) and *Methanococcus voltae* (DSM 1537) were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany (13). *Methanobacterium bryantii* MoH was a gift of R. S. Wolfe. *Methanobacterium thermoautotrophicum* Marburg and Δ H were from this laboratory. The media used in this study are described in Table 1. Cultivation was mostly carried out in either 250-ml serum bottles (no. 223950; Wheaton Scientific, Millville, N.J.) sealed with cutoff no. 2 black rubber stoppers and aluminum seals or 100-ml serum bottles (Wheaton no. 223747) sealed with black rubber stoppers (no. 2048-11800; Bellco Biotechnology, Vineland, N.J.) and aluminum seals. Bottles of media were made anaerobic by evacuation and flushing with H₂-CO₂ (80:20 [vol/vol]), cysteine was added as the sole reducing agent to each bottle, and the bottles were autoclaved under protective covers at 121°C for 20 min (10).

Determination of MICs of copper. Cultures were grown in stoppered serum tubes (26.5 ml, total volume; no. 2048-00150; Bellco Glass, Vineland, N.J.) containing 5 ml of medium with different copper concentrations and 4 mM cysteine as the sole reducing agent. All cultures were grown in isolate medium (IM) supplemented with 5 mM acetate (after several transfers to adapt cells to the medium), except for *Methanococcus voltae*, which was grown in medium IV. The tubes were inoculated (10% [vol/vol]) to give equal culture absorbances. Methane production was determined by gas chromatography (19). The MIC was defined as the lowest concentration of CuSO₄ at which cells did not produce more than 5% of the methane produced by the control cultures.

Isolation of copper-resistant methanogens from soil samples. Soil samples were taken from a site of the White Pine Copper Co. (White Pine, Mich.), which mines and refines ore containing both native copper and copper sulfide. At this location, two ponds are used to treat wastes; the first accepts cooling water from the plant and water from underground mining, as well as sewage from the town of White Pine, and empties into the second pond, which receives copper-processing water directly. Eight samples were taken at the copper-mining area: three from the edges of the copper mine wastewater ponds, one at the edge of a stream exiting the second pond, and four from nearby fields. Sterile plastic bottles were filled with soil and sealed with screw caps and Parafilm M.

Each soil sample (20 ml) was transferred in an anaerobic glove bag to 100-ml anaerobic sterile serum bottles with disposable 5-ml syringes with ends made blunt with a razor blade. The bottles were removed from the glove bag after being sealed with stoppers and aluminum caps. Each bottle was flushed with H₂-CO₂ for 5 min, and the pressure was adjusted to 1 atm (1 atm = 101.29 kPa) (gauge). Soil in each bottle was resuspended in 30 ml of anaerobic sterile medium II, and an anaerobic antibiotic mixture was added to give a concentration of 100 U of penicillin G per ml and 50 µg of oleandomycin per ml. Anaerobic filter-sterilized CuSO₄ was added to a concentration of 1.0 mM for selective pressure during isolation. The bottles were incubated standing at 37°C and mixed two times per day by hand; methane production was checked every 2 to 3 days. The bottles were flushed and pressurized with H₂-CO₂, depending on

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TABLE 1. Compositions of basal media used to grow methanogens, to determine MICs of copper, or to isolate and characterize methanogens from soil samples^a

Medium	Concn													
	KH ₂ PO ₄ (mM)	K ₂ HPO ₄ (mM)	NH ₄ Cl (mM)	NaCl (mM)	NaHCO ₃ (mM)	Sodium acetate (mM)	MgCl ₂ · 6H ₂ O (mM)	MgSO ₄ · 7H ₂ O (mM)	CaCl ₂ · H ₂ O (mM)	KCl (mM)	Fe(NH ₄) ₂ (SO ₄) ₂ (μM)	Yeast extract (g/liter)	Casamino Acids (g/liter)	
I	3.09	1.26	18.70	5.13			0.39		0.41			0.5	0.5	
II	3.09	1.26	13.08	10.27		20.00	0.19		0.21			0.5	0.5	
III	15.27	9.73	40.00	5.00			0.19							
IV		0.80	4.67	308.01	59.52	12.19	20.95	17.28	0.95	4.49	4.87	0.5	0.5	
IM	3.09	1.26	6.54	5.00			0.10		0.10					

^a L-Cysteine · HCl (4 mM) was used as the sole reductant. All media contained 1.5 μM resazurin and 10 ml of the *Methanococcus* mineral elixir (I, II, IV, and IM) or the *Methanobacterium* mineral elixir (34) per liter. All media except medium III also had 10 ml of vitamin elixir (34) per liter. The pH of each medium was adjusted to 6.8 with Na₂CO₃ while under a 20% CO₂ gas phase at atmospheric pressure. The following media were used as indicated: type I, for initial cultivation of *Methanobacterium bryantii* MoH; type II, for initial cultivation of *Methanobacterium formicicum* and for soil enrichments; type III, for initial cultivation of *Methanobacterium thermoautotrophicum* Marburg and ΔH; type IV, for cultivation and for determination of the MIC of copper for *Methanococcus voltae*; and type IM, for characterization of the isolate and, with 5 mM sodium acetate, for determination of the MIC of copper for the isolate and known methanogens except *Methanococcus voltae*.

the methane production. The first culture transfers were made into bottles with the same but fresh medium, except containing 100 μg of cephalosporin C per ml and 50 μg of kanamycin A per ml, but the transfer time was decided by culture behavior.

After three transfers, a modified roll tube method (17), with the use of serum tubes with medium II containing 1% agar and 10-fold dilutions of the enrichment cultures, was used to isolate colonies. After completion of the transfers, each completed roll tube was flushed, pressurized to 1.3 atm with H₂-CO₂, and incubated at 37°C. Methane production was monitored, and the tubes were fed with gas depending on performance. Colonies were transferred in the glove bag to serum tubes containing medium II with antibiotics and copper. Sterile Pasteur pipettes, the tips of which had been bent in a flame and the other ends of which had been plugged with cotton, were used to pick colonies. Inoculated tubes were treated as described above, and the cultures were transferred several times to fresh medium. The roll tube method was repeated once for the culture with the highest level of methane production among the first colony isolate cultures, to assure a pure culture.

Nutritional studies of isolated methanogen. Medium II without acetate was used to examine requirements for yeast extract or Casamino Acids. IM (Table 1) was used to test the isolate for the use of acetate, methanol, formate, or H₂. All media, except that for testing H₂, were made anaerobic with N₂-CO₂ gas (80:20 [vol/vol]). Before medium sterilization, acetate, methanol, or formate was added from an anaerobic stock solution (pH 7) to anaerobic medium in serum tubes, to achieve concentrations of 0.05, 0.1, or 0.2 M.

Electron microscopy. A transmission electron microscope (TEM, Hitachi model H-600) and a scanning electron microscope (SEM, Hitachi model S-4000) were used to examine the isolate at the University of Iowa Electron Microscopy Facility. Samples were prepared with glutaraldehyde and osmium as described previously (35).

Antigenic fingerprinting of the isolate. The partial antigenic fingerprint was determined by indirect immunofluorescence and a quantitative slide immunoenzymatic assay with a panel of S probes for rod-shaped methanogens, *Methanobrevibacter smithii* PS and ALI; *Methanobrevibacter ruminantium* M1; *Methanobrevibacter arboriphilicus* DH1, A2, and DC; *Methanobacterium thermoautotrophicum* GC1 and ΔH; *Methanobacterium formicicum* MF; and *Meth-*

anobacterium bryantii MoH and MoHG (23, 24). The isolate was cultivated in one of two ways: with 1.0 mM CuCl₂ or without copper. Cells were harvested from 30 ml of culture by centrifugation (10,000 × g, 5 min) at 4°C, and the pellet was resuspended in 1 ml of saline containing 1.5% (wt/wt) formaldehyde and processed for immunological analysis.

Preparation of genomic DNA from the isolate. The isolate was cultivated with H₂-CO₂ as the growth substrate in a fermentor. The cell pellet (4.6 g) was suspended in Tris-EDTA buffer and subjected to three cycles of freezing-thawing; then, pronase was added at 0.2 mg/ml to the cell suspension, and the suspension was incubated at 37°C for 2 h. Protease K was then added at 0.075 mg/ml with calcium chloride (0.003%) and sodium dodecyl sulfate (2.5%), and the mixture incubated for 3 h at 37°C. The remaining steps were performed as described previously (41).

16S rRNA gene cloning and sequencing. The 16S rRNA gene was amplified from genomic DNA by PCR. Two primers were used to facilitate cloning of PCR products: primer 1510Pst and primer 124Bam (14, 32). The PCR product was subcloned in pUC18 with *HincII* and *Escherichia coli* DH5α (41). Both strands were sequenced for about 300 nucleotides by the dideoxy chain termination method (37).

RESULTS

MICs for known methanogens. CuSO₄ was used to define MICs for the methanogens listed in Table 2. The two strains of *Methanobacterium thermoautotrophicum* were the most sensitive, and *Methanobacterium bryantii* was the most resistant. None of these methanogens could grow in medium containing 1.0 mM cupric salt, and even the most resistant strains were significantly affected by 0.2 to 0.5 mM cupric salt. The 2-day MICs were, in some cases, lower than those at 6 days because of a lag caused by the Cu. Thus, 1 mM CuSO₄ was

TABLE 2. Effect of CuSO₄ on methanogens as determined by the MIC and response to copper at concentrations below the MIC

Organism	MIC ^a (mM) on day:		Methane production (% of control with no added copper) at day 6 with addition of:		
	2	6	0.2 mM Cu	0.5 mM Cu	1.0 mM Cu
<i>Methanobacterium bryantii</i> MoH	1.0	1.0	50	39	0
<i>Methanobacterium formicicum</i>	0.5	1.0	72	62	0
<i>Methanococcus voltae</i>	0.5	0.5	14	0	0
<i>Methanobacterium thermoautotrophicum</i>					
Marburg	0.05	0.1	0	0	0
ΔH	0.05	0.1	0	0	0
<i>Methanobacterium bryantii</i> BKYH (isolate)	~1.8 ^b	~1.8	ND ^c	100	100

^a No growth is defined here as <5% of methane production in the control with no added copper.

^b Since no turbidity was seen in the medium containing 1.5 mM added cupric salt, but slight turbidity was seen at 1.8 mM CuSO₄, the total soluble copper (free Cu²⁺ plus soluble chelated copper) may be between 1.5 and 1.8 mM.

^c ND, not determined but should be ~100%.

FIG. 1. SEM pictures of isolate cultivated with H_2 - CO_2 in medium II containing 1.5 mM cupric salt. The panel on the right is a five-times-greater magnification of the area outlined in the panel on the left. Bar, 4 μ m.

used for the isolation of copper-resistant methanogens from soil samples.

Isolation and properties of a copper-resistant methanogen. After 8 days of soil enrichments, the first transfer was carried out for the sample taken at the edge of the stream exiting the waste ponds at the White Pine Co. site, which had shown the highest level of methane production. After several transfers, the roll tube method gave 28 colonies, which were picked and cultured; 17 grew and showed similar methane production rates and cell shapes with the phase-contrast microscope. After several transfers, the roll tube method was repeated with 1 of the 17 cultures, from which 1 culture was used for further study.

The isolated methanogen had a conventional methanobacterium-like rod shape in medium without copper, and at higher copper concentrations, the cells were long and filamentous and showed a twisted cellular bundle shape that has not previously been reported for methanogens (Fig. 1). The 2-day and 6-day MICs were ~ 1.8 mM, as shown in Table 2. The isolate grew on H_2 - CO_2 or formate but not on acetate or methanol. Growth on formate was slower than that on H_2 - CO_2 . Acetate, Casamino Acids, or yeast extract was not required for growth.

The isolate formed a floating mat on the surface of liquid medium in standing cultivation with H_2 - CO_2 . This phenomenon was dependent on the presence of H_2 - CO_2 gas in the bottle but was not dependent on copper. The cell mat disappeared when the H_2 - CO_2 content in the bottles decreased because of methanogenesis; the mat then reformed on the surface after H_2 - CO_2 was reintroduced. This cycle was reproduced several times in the same bottle. This finding suggests that cell mat formation is dependent on an energy source.

As shown in Fig. 2, SEM revealed that floating mat cells stuck together side by side and tip to tip. TEM did not show evidence of gas vacuoles in floating cells (Fig. 3) but showed one electron-dense layer close to the cytoplasmic membrane, as expected for a type 1 envelope (18).

Antigenic fingerprinting. The isolate was antigenically fingerprinted at 11 positions: 1 and 10, *Methanobrevibacter smithii* PS and AL1, respectively; 2, *Methanobacterium formicicum* MF; 4 and 5, *Methanobacterium bryantii* MoH and MoHG, respectively; 8, *Methanobrevibacter ruminantium* M1; 9, 21, and 22, *Methanobrevibacter arboriphilicus* DH1, AZ, and DC, respectively; and 11 and 12, *Methanobacterium thermoautotrophicum* GC1 and Δ H, respectively. The probes for *Methanobacterium bryantii* (positions 4 and 5) showed a spotty reaction, and reactions were not seen at the other positions.

16S rRNA gene of isolated methanogen. The gene encoding 16S rRNA was amplified from genomic DNA by PCR. As a positive control, the 16S rRNA gene of *Methanobacterium thermoautotrophicum* Δ H was amplified at the same time by PCR. Both PCR products migrated at about 1.3 kb on an agarose gel (data not shown). The PCR product for the isolate was cut into three pieces by *Bam*HI or *Pst*I, but the PCR product treated with *Hinc*II showed almost the same size as the untreated PCR product on an agarose gel and thus was selected for use in subcloning. Sequencing was carried out for the cloned 16S rRNA gene in pUC18 with the two primers used for PCR. About 300 nucleotides were read in both directions, and the sequences were compared with the sequences in the GenBank database with the Blast program from the National Center for Biotechnology Information. The segments with the highest levels of similarity included only methanogens. The *Methanobacterium bryantii* MoH 16S rRNA gene had the highest level of identity, 97%, in both strands. The sequence identity of *Methanobacterium formicicum* ATCC 33274 ranked second in the minus strand (94%) and third in the plus strand (90%).

DISCUSSION

The MIC of copper for the isolated methanogen (estimated at ~ 1.8 mM; see Table 2, footnote b) was ~ 2 - to 36-fold higher than the MICs for the known methanogens tested in our work;

FIG. 2. SEM pictures of floating cell mat. The isolate was cultivated with H_2 - CO_2 in medium II without shaking. The SEM sample was prepared from a floating cell mat. (A) The cell mat shows a porous structure. Magnification, $\times 500$. Bar, $30\ \mu m$. (B) Area outlined in panel A at a higher magnification ($\times 4,000$). Bar, $4\ \mu m$.

even the most-resistant known methanogens were significantly affected by 0.2 to 0.5 mM copper, but the isolate was routinely grown in 1 mM Cu. Except for that for *Methanococcus voltae*, all MICs were determined in the same medium, at the same pH, and with the same inoculum size, and therefore the initial Cu concentrations to which the cells were exposed were identical. In aerobes that carry specific copper resistance plasmids, the plasmid typically provides the cell with a two- to fourfold-higher MIC (3), which is similar to the difference between the laboratory strains of *Methanobacterium bryantii* and *Methanobacterium formicicum* and our isolate. Although the mechanism by which our isolate resists copper toxicity is unknown, the isolate, when exposed to 1 mM Cu, does excrete a glycoprotein (CRX) in levels fourfold-higher than those in cultures with no added Cu; this protein is not produced by other methanogens that have been examined (20) and is thus a reasonable target for further resistance mechanism study. In the work by Ahring and Westermann (2) with medium similar to that used in this study (e.g., 4 mM cysteine instead of sulfide), MICs of 0.08 and 0.4 mM copper for a thermophilic acetoclastic methanogen (most likely, a *Methanosaeta* sp.) and *Methanobacterium thermoautotrophicum*, respectively, were reported. Work

on methanogen heavy-metal toxicity by others (16, 33) is not directly comparable, since they used sulfide as a reductant, and it is known that sulfide has a major effect on metal precipitation and on apparent MICs (1, 21).

Kandler and König (18) have classified archaeobacteria into four cell envelope types based on their TEM profiles and cell wall composition. Type 1 envelopes have one electron-dense layer with various degrees of homogeneity, which closely adjoins the cytoplasmic membrane. Types 2, 3, and 4 have significantly different TEM appearances. Since our isolate has a rod shape and a type-1 cell wall, it was most likely either a species of *Methanobrevibacter* or *Methanobacterium* (6). The positive reaction of the isolate with the S probes for *Methanobacterium bryantii* was clear but distributed in spots, indicating partial immunologic relatedness, possibly due to spaced distribution of the epitope(s) recognized by the probes. On the 16S rRNA-gene sequence comparison, the isolate is slightly closer to *Methanobacterium bryantii* than to *Methanobacterium formicicum*. However, the isolate utilized formate as a sole energy and carbon source as *Methanobacterium formicicum* does (6), although known *Methanobacterium bryantii* strains cannot utilize formate. On the basis of the electron micro-

FIG. 3. TEM pictures of the isolate. The isolate was cultivated with H_2 - CO_2 in medium II without shaking. The TEM sample was prepared from a floating cell mat or culture near the bottom in a culture bottle. (A) Cells taken from liquid phase. Bar, 0.3 μm . (B) Cells taken from the cell mat. Bar, 0.4 μm .

scopic ultrastructure, antigenic fingerprint, substrate use, and 16S rRNA gene sequence data, we conclude that the isolate is a new strain of *Methanobacterium bryantii* with the ability to utilize formate and have named the isolate *Methanobacterium bryantii* BKYH; this culture has been deposited at the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH as culture no. 9575.

The isolate became more filamentous as copper concentrations increased. This kind of morphological change which is not dependent on copper has been reported for *E. coli* (5), *Bacillus subtilis* (28), and *Rhizobium meliloti* (25), in which the change is caused by mutation or overexpression of genes that encode proteins that play a role in cell division: *sulA*, *ftsZ*, *minCD*, or *minCDE* (5, 22). When FtsZ activities are inhibited, *E. coli* becomes longer or makes minicells (42). FtsZ is a guanine nucleotide-binding protein (31) with homology to tubulin, which functions in eukaryotic cell division (22), and is also involved in cell division. Tubulin, like FtsZ, has a binding site for a guanine nucleotide, and there are several Ca^{2+} bind-

ing sites: one high-affinity site and several low-affinity sites. Calcium binding to the high-affinity site might be inhibited by Mg^{2+} (39). Possibly, Cu^{2+} could inhibit an FtsZ-like protein in the isolate, as Mg^{2+} does with tubulin, and make cells filamentous. The Western blot (immunoblot) results with antibodies against *E. coli* FtsZ protein show that this protein is highly conserved among eubacteria (7), and it would be of interest to determine if an FtsZ-like protein is present in the isolate and if it plays any role in copper-dependent morphological changes.

The phenomenon of cell intertwining has been reported for a *B. subtilis* mutant (28, 29) in which intertwining is so tight that microfibers can be seen with the eyes. Mendelson (29) also showed that the cell surface of *B. subtilis* grown in web form could nucleate precipitation of added soluble metals (Ca^{2+} , Fe^{3+} , and Cu^{2+}) and that cells could be drawn into a fiber after being mineralized with the metals. These phenomena were interpreted as the cell walls acting as ion exchangers (26), with metal ions such as Ca^{2+} , Fe^{3+} , or Cu^{2+} binding and cells

adhering to each other (4). This interaction might explain the behavior of our isolate in high-copper medium, in which cells became long and intertwined. Copper may first induce a filamentous cell shape and subsequently act like glue on the cell surface, resulting in intertwined cells.

The isolate showed another novel feature, cell mat formation on the surface of liquid standing cultures grown with H_2 - CO_2 . This feature has not previously been reported for methanogens, but a similar phenomenon called lamina formation has been described for *Methanosarcina mazei* grown on trimethylamine in medium relatively high in Ca^{2+} and/or Mg^{2+} levels (27). Gas vacuoles were not evident in the thin section of the mat cells, and the cells' insides were unremarkable (Fig. 3). The light regions in the cytoplasm (Fig. 3) are regions of chromosome condensation caused by chemical fixatives, especially uranyl acetate. The most apparent difference between the "floaters" and the "sinkers" was the contrast of the cell walls. The sinkers had more deeply stained walls, suggesting that they have more anionic sites available for heavy metal (osmium) complexation during fixation. This means that sinkers could have more hydrophilic surfaces than floaters have. Since the cell walls of the floaters did not bind as much stain, they could be more hydrophobic. This property (unwettability) could account for their tendency to float. However, the possibility of gas vacuoles in the floaters still can not be ignored, since centrifugal force during sample preparation might break the gas vacuoles (44). Whether unwettability, gas vacuoles, or something else causes the cells to float, the question of why this phenomenon occurs is still left. One possibility is tactic response. Chemotaxis in methanogens has been reported for *Methanospirillum hungatei* (30) and *Methanococcus voltae* (40) with several attractants (acetate, formate, isoleucine, leucine, or histidine but not H_2 - CO_2). The relationship of mat formation to H_2 - CO_2 pressure in the culture suggests that, in some unknown way, a tactic response to H_2 may occur.

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