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Methylmercury Resistance in *Desulfovibrio desulfuricans* Strains in Relation to Methylmercury Degradation

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Two strains of *Desulfovibrio desulfuricans*, one known to synthesize monomethylmercury from ionic mercury, were grown to determine methylmercury toxicity and for comparison with an anaerobic strain of *Clostridium pasteurianum*, a H_2 producer, and with the broad-spectrum mercury-resistant *Pseudomonas putida* strain FB-1, capable of degrading 1 μg of methylmercury to methane and elemental mercury in 2 h. The CH_3HgCl resistance of *D. desulfuricans* strains was 10 times that of *P. putida* FB-1 and 100 times that of *C. pasteurianum*. The methylmercury resistance of *D. desulfuricans* was related to the disappearance of methylmercury from cultures by transformation to dimethylmercury, metacinnabar, methane, and traces of ionic mercury. During a 15-day experiment the kinetics of the two volatile compounds dimethylmercury [$(CH_3)_2Hg$] and methane were monitored in the liquid by a specific new technique with purge-and-trap gas chromatography in line with Fourier transform infrared spectroscopy and in the headspace by gas chromatography with flame ionization detection. Insoluble metacinnabar (cubic HgS) of biological origin was detected by X-ray diffractometry in the gray precipitate from the insoluble residue of the pellet of a 1-liter culture spiked with 100 mg of CH_3HgCl. This was compared with a 1-liter culture of *D. desulfuricans* LS spiked with 100 mg of $HgCl_2$. In a further experiment, it was demonstrated that insoluble, decomposable, white dimethylmercury sulfide [$(CH_3Hg)_2S$] formed instantly in the reaction of methylmercury with hydrogen sulfide. This organomercurial was extracted with chloroform and identified by gas chromatography in line with mass spectrometry. The *D. desulfuricans* strains were resistant to high concentrations of methylmercury because they produced insoluble dimethylmercury sulfide, which slowly decomposed under anaerobic conditions to metacinnabar and volatilized to dimethylmercury and methane between pHs 6.2 and 6.5 for high (4.5-g · liter^{-1}) or low (0.09-g · liter^{-1}) sulfate contents. Methane was produced from CH_3HgCl at a lower rate than by the broad-spectrum Hg-resistant *P. putida* strain FB-1.

Mercury and its compounds are among the most toxic pollutants for microorganisms. The toxicity of metals also depends on redox potential, which affects metal speciation. Inorganic mercury species, for example, are more toxic in aerated ecosystems, whereas in estuarine and marine anoxic sediments soluble ionic mercury is transformed by H_2S to harmless and insoluble HgS (12, 18). Methylmercury toxicity has not been sufficiently investigated under anaerobic conditions. Rudrik et al. (33) found a variable resistance to inorganic and organic mercury compounds in strictly anaerobic bacteria (*Bacteroides ruminicola* and *Clostridium perfringens*) isolated from clinical settings and sewage. The mercury resistance of these isolates was neither inducible nor plasmid mediated. Pan-Hu et al. (29) isolated strain T-2 of *Clostridium cochlearium*, the methylmercury-decomposing activity of which is harbored in a plasmid associated with H_2S production, but mercuric reductase activity has never been detected in this strain.

Rowland et al. (32) and then Craig and Bartlett (15) demonstrated that H_2S chemically volatilized methylmercury to dimethylmercury sulfide, which decomposed to dimethylmercury and HgS. Wood (35) proposed that this disproportionation could be carried out biologically by the sulfate-reducing *Desulfovibrio* species. Nevertheless, dimethylmercury is occasionally detected in ecosystems. Dimethylmercury was first detected by Jensen and Jernelöv (24) in aquarium sludge and in rotten fish spiked with $HgCl_2$. Imura et al. (23) demonstrated that under anaerobic conditions methylcobalamine chemically methylated $HgCl_2$ to monomethylmercury, but traces of dimethylmercury suggested that the latter was probably the first product of this reaction. In laboratory studies (6) and environmental assessments (1) of the early 1970s, dimethylmercury was detected only as total methylmercury in acidified samples. Since then, it became common opinion that methylmercury was the sole organomercurial synthesized by bacteria in aquatic environments.

There were few reports (7, 13) of dimethylmercury detection in situ or under controlled conditions until recently, when dimethylmercury was detected under the subthermocline of the Pacific Ocean (25) and in mangrove sediment cores (31). The finding of dimethylmercury in different environments is due mostly to the development of specific and sensitive methods (9, 21, 25). Evidence of monomethylmercury formation by sulfate-reducing bacteria via a cobalamine-like pathway (5, 11) and of dimethylmercury in natural environments raised the question of the primary organomercurial species formed by bacteria. The search for an answer is not just an analytical exercise, but opens new fields of investigation of the microbial origin of organomercurials in the environment.

The aim of this study was to demonstrate that two different axenic cultures of strains of the sulfate-reducing bacterium *Desulfovibrio desulfuricans* were resistant to high concentrations of methylmercury by virtue of a mechanism different from that of broad-spectrum Hg-resistant bacteria. Methylmercury toxicity in anaerobic and aerobic bacteria

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was compared in order to demonstrate the high resistance of sulfate-reducing bacteria. Identification of mercury species was carried out with specific and sophisticated techniques to demonstrate the possible mechanism of monomethylmercury resistance in sulfate-reducing bacteria.

MATERIALS AND METHODS

Bacterial cultures. Experiments on the microbial transformation of methylmercury and $HgCl_2$ were performed with two strains of *D. desulfuricans*. Strain LS methylates inorganic mercury to methylmercury (5, 11) and was kindly donated by R. Bartha; the other strain, API, from the Analytab Products collection, was kindly provided by G. Olson. Both strains were cultured routinely in Postgate medium C containing, per liter, 0.5 g of KH_2PO_4, 1 g of NH_4Cl, 4.5 g of $NaSO_4$, 0.06 g of $CaCl_2 \cdot 6H_2O$, 0.06 g of $MgSO_4 \cdot 7H_2O$, 6 g of sodium pyruvate, 1 g of yeast extract, 0.3 g of sodium citrate, and 0.1 g of NaCl. The medium was finally adjusted to pH 7.5 with NaOH solution.

Another anaerobic microorganism, *Clostridium pasteurianum*, a H_2 producer, was kindly supplied by G. Vallini. It was routinely grown in synthetic medium containing, per liter, 40 g of sucrose, 4 ml of $MgCl_2 \cdot 6H_2O$ (7.4% solution), 1 ml of 20% NaCl solution, 0.2 ml of 10% $Na_2Mo_4 \cdot 2H_2O$ solution, 1.5 g of $CaCO_3$, 0.1 ml of *p*-aminobenzoic acid (100 $\mu g \cdot ml^{-1}$), 2.0 ml of Na_2SO_4 (7.1% solution), and 16 ml of NH_4Cl (20% solution).

Hg-sensitive strain KT 2440 of *Pseudomonas putida* was kindly supplied by B. Cameron (10). The broad-spectrum Hg-resistant strain FB-1 of the same species was from our collection. This strain degrades methylmercury, ethylmercury, and phenylmercury (2, 3) by means of organomercurial lyase. Both strains were routinely grown in Nelson medium designed for mercury toxicity tests (26), containing, per liter, 5 g of Casamino Acids, 2 g of D-glucose, 1 g of yeast extract, 0.1 g of $MgSO_4 \cdot 7H_2O$, and 0.9 g of NaCl.

MIC tests. The sulfate-reducing strains were grown in two different media: Postgate C, with high concentrations of sulfates (4.5 $g \cdot liter^{-1}$) as described above for routine growth, and Postgate D, with low concentrations of sulfates (0.090 $g \cdot liter^{-1}$) as impurities. Medium D contained, per liter, 0.5 g of KH_2PO_4, 1 g of NH_4Cl, 0.1 g of $CaCl_2 \cdot 2H_2O$, 1.6 g of $MgCl_2 \cdot 6H_2O$, 1 g of yeast extract, 3.5 g of sodium pyruvate, and 0.1 g of NaCl, adjusted to pH 7.5 with NaOH solution. A 0.5-ml aliquot of a 2-day-old culture of *D. desulfuricans* in Postgate C medium was inoculated in 50 ml of fresh Postgate C and D media amended with different concentrations of CH_3HgCl (0.25 to 50 $\mu g \cdot ml^{-1}$) from a stock solution (10 g of $CH_3HgCl \cdot liter^{-1}$ as total Hg in ethanol). Cultures to which CH_3HgCl had not been added were used as controls. The bacteria were incubated for 14 days and counted (bacteria per milliliter) in each serum bottle by using a Petroff-Hausser cell-counting chamber with a differential interference contrast microscope (Zeiss Axiophot). The cells were first sampled (0.5 ml) with a syringe and then fixed with 4% paraformaldehyde in PIPES [piperazine-*N*,*N*'-bis(2-ethanesulfonic acid)] buffer solution at pH 7.4. The growth of sulfate-reducing bacteria was checked on the basis of pH measured after 15 days of incubation.

The MIC of methylmercury for *C. pasteurianum* was determined in synthetic medium by adding the same serial concentrations of CH_3HgCl and counting cells per milliliter as for *D. desulfuricans* cultures.

The MICs for *P. putida* strains were determined in Nelson medium by adding 0.1 to 5.0 μg of CH_3HgCl ml^{-1} as total Hg to 10 ml of culture diluted 1:100. Growth was tested after 18 h of incubation at 28°C in a rotary drum by determining the A_{600} with a spectrophotometer (UV-visible light; Shimadzu). The number of cells per milliliter and optical density were normalized with respect to percentage of growth for the purpose of comparison.

Dimethylmercury detection. Dimethylmercury was detected in *D. desulfuricans* cultures grown in different media (Postgate C and D). Bacterial suspensions of cultures spiked with different concentrations of CH_3HgCl at a constant time (after 15 days) and/or of active cultures spiked with 100 $\mu g \cdot ml^{-1}$ at different times were sampled with a syringe, and 5 ml of sample was transferred to a 10-ml glass vessel without adding any chemical. The vessel was sealed to the purge-and-trap unit (Chrompack), and the suspension was purged with N_2 for 5 min at 80°C as reported previously (20). The volatile dimethylmercury was trapped in a column at −120°C, released after heating the column to 250°C, and automatically injected into a gas chromatograph (Carlo Erba model HRGC 5300 Mega series) in line with a Fourier transform interferometer (Nicolet 20 SXB) equipped with an optical bench accessory. A CP-Sil 8 fused silica column (50 m by 0.53 mm; film thickness, 2 μm; Chrompack) was operated isothermally at 100°C with a 10-ml/min N_2 flow rate. The infrared light pipe (15-cm length) was heated to 120°C. Dimethylmercury was identified by its retention time and infrared spectrum. Results were linear up to 100 μg, and the sensitivity of the test was 0.8 μg of dimethylmercury. The retention time and peak area of dimethylmercury were determined with a flame ionization detector.

Methylmercury detection. The CH_3HgCl residues in *D. desulfuricans* cultures were quantitated at different times. The sample was boiled for 10 min in 10 ml of 1 N HCl, and the CH_3HgCl was extracted four times with 1 ml of toluene. The solvent layer was cleaned with anhydrous Na_2SO_4, and 1 μl was injected into a gas chromatograph (Carlo Erba model HRGC 5160) equipped with an electron capture detector. The column (HP-1 Methylsilicone; 30 m by 0.53 mm [internal diameter]; film thickness, 2.65 μm) was operated isothermally at 120°C (19). Five replicate analyses gave a coefficient of variation of 7.3%.

Methylmercury was converted to methylmercury hydride by adding sodium boron hydride to the sample and determining its spectrum by purge-and-trap gas chromatography (GC) in line with Fourier transform infrared spectroscopy (20).

Ionic mercury detection. Ionic mercury was detected in *D. desulfuricans* cultures at different times after removal of 1 ml of the methylmercury chloride residue in the aqueous phase and after repeated extractions of 1 ml of toluene. The methylmercury-free aqueous phase was mineralized with 1 ml of concentrated HCl for 1 h at 80°C. The ionic Hg was detected in 1 ml of sample by reduction to elemental mercury with 12% $SnCl_2$ in 1 N H_2SO_4 solution and quantitated by flameless atomic absorption spectrophotometry (Perkin-Elmer 300S spectrophotometer). Five replicate analyses gave a coefficient of variation of 5.3%.

Elemental mercury detection. Elemental mercury was detected in the headspace of all *D. desulfuricans* strain cultures. One milliliter of headspace gas from serum bottles was collected with a gas-sampling syringe (Supelco) and injected into the atomization cell of the atomic absorption spectrophotometer. Detection of Hg(0) was performed by injecting different volumes (from 0.1 to 1.0 ml) of headspace gas from a sealed vial containing metallic mercury in drops. Calcula-

tions were made on the basis of Hg vapor pressure at room temperature (8).

Methane detection. The volatile hydrocarbon methane was detected in the headspace of *D. desulfuricans* cultures spiked with CH_3HgCl. An aliquot (0.25 ml) was taken with a gas-sampling syringe and injected into a gas chromatograph (Hewlett Packard model 5890A) equipped with a flame ionization detector. The methane content was also confirmed by its spectrum, obtained by injecting 1 ml of headspace gas into a GC-Fourier transform infrared spectroscopy apparatus (3). Different volumes of 1% methane (Supelco) in nitrogen were diluted in a gas-sampling flask (250 ml), and 0.25 ml of standard was injected to calibrate the method. Five replicates of the same sample gave a coefficient of variation of 3.1%.

Identification of mercury species in cultures. Two liters of medium C (with sulfates), anaerobically prepared, was split in two 1-liter bottles and inoculated with a 10% inoculum of *D. desulfuricans* LS. One bottle was spiked with 100 mg of CH_3HgCl as Hg, and the other was spiked with 100 mg of $HgCl_2$ as Hg. As soon as CH_3HgCl was added to the culture, a white plume formed, whereas with $HgCl_2$ a black plume formed. After 15 days of incubation at 28°C the two samples were centrifuged at 4,200 × *g* for 25 min. Total Hg was quantitated in the supernatant by graphite furnace atomic absorption spectrophotometry after acidification of the sample. The two pellets recovered in blood graduated test tubes showed volumes of 1.0 ml for the sample with CH_3HgCl and 1.3 ml for that with $HgCl_2$. The cell dry weight of the pellets was measured in a subsample (0.2 ml) by heating the sample to 60°C until a constant weight was reached. The pellets were previously washed in N_2-saturated PIPES buffer solution and centrifuged again while keeping them under N_2 as much as possible. Two subsamples were used to determine the dry weight of the cells. Aliquots (0.2 ml) of two pellets were boiled for 10 min in 10 ml of 1 N HCl solution. Then the samples were centrifuged and extracted four times with 1 ml of toluene for organic mercury. The aqueous layers were analyzed for soluble forms of inorganic mercury (19). The gray precipitates in the CH_3HgCl-spiked sample and the dense black precipitates in the $HgCl_2$-spiked ones were weighed after drying the samples at 60°C until a constant weight was reached. Then HgS was identified in both samples by X-ray diffractometry (Siemens D500) from the highest peak of relative intensity and other specific peaks of cubic crystals of metacinnabar.

Dimethylmercury sulfide [$(CH_3Hg)_2S$] was found in a separate experiment in which 10 mg of CH_3HgCl in 10 ml of aqueous solution was mixed with 10 ml of a 2-day *D. desulfuricans* culture. The white precipitate that instantly formed was dissolved in 2 ml of chloroform, extracted with a separator funnel, and then concentrated to 1 ml. One microliter was injected into a gas chromatograph in line with a mass spectrometer (HP 5895) equipped with a fused silica column (25 m by 0.2 mm) of phenyl-methyl silicone (5%) (0.33-μm film thickness). The injection port was at 270°C, and the analysis was performed with a temperature program from 100 to 270°C at a rate of 25°C/min.

RESULTS

Despite evident differences in the complex agents of each medium, the size of the bacterial inoculum, the times of growth, and the number of cells per milliliter, the MIC tests for aerobic and anaerobic bacteria showed that methylmercury was 10 times less toxic for sulfate-reducing bacteria than for aerobic broad-spectrum mercury-resistant *P. putida* FB-1 (Fig. 1). This strain is known to completely degrade 1 μg of methylmercury to methane and elemental Hg(0) in 2 h (3). Moreover, *P. putida* FB-1 was more resistant than methylmercury-sensitive *P. putida* KT 2440 and even more resistant than *C. pasteurianum*.

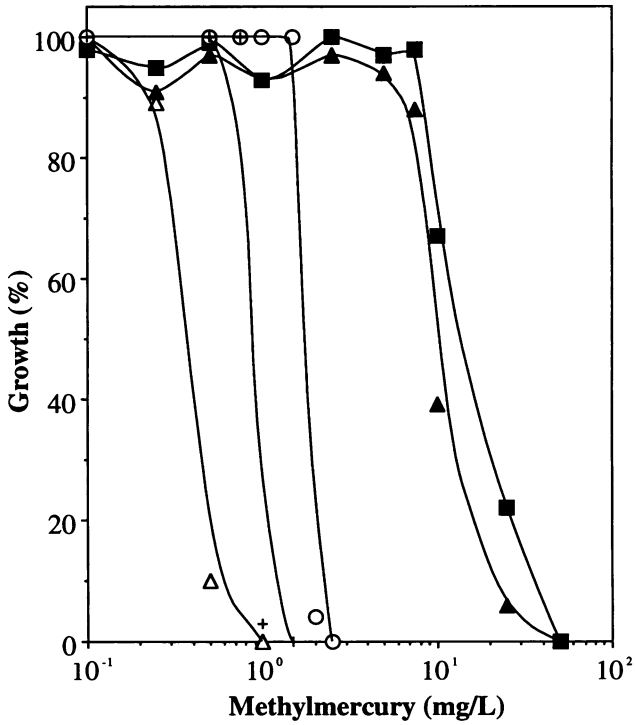

FIG. 1. MIC tests for *D. desulfuricans* LS (▲) and API (■), *P. putida* FB-1 (○) and KT 2440 (+), and *C. pasteurianum* (△). Cultures were spiked with the different concentrations of CH_3HgCl at the start of the experiment 5 min after the inoculum was administered.

Causes of the methylmercury resistance of the two sulfate-reducing strains were investigated in samples amended with CH_3HgCl by identification of Hg species in the headspace gas, liquid cultures, cell pellet, and precipitates. The half time of 100 μg of CH_3HgCl ml^{-1} in the liquid cultures calculated over a 15-day period was 16.1 days. The degradation was entirely due to its transformation, as there were no losses by volatilization. All serum bottles were sealed so that Hg could not be lost but could only be transformed.

The formation of dimethylmercury in liquid cultures was evident in the presence of both high and low concentrations of sulfates. The difference between dimethylmercury production levels with 4.5 and 0.090 g of sulfate $liter^{-1}$ was not very significant. The formation of dimethylmercury from monomethylmercury in *D. desulfuricans* cultures increased exponentially in relation to days (*d*) of incubation both with high sulfate content ($0.39 \cdot 10^{0.12d}$) and with low sulfate content ($0.36 \cdot 10^{0.11d}$).

Formation of $(CH_3)_2Hg$ in *D. desulfuricans* LS culture was positively correlated ($R^2 = 0.982$) with increasing concentrations of CH_3HgCl up to 10 μg/ml after 15 days of incubation at 28°C (Fig. 2). The ratio of $(CH_3)_2Hg$ formation was 0.16 μg · ml^{-1} · μg of CH_3HgCl^{-1} spiked in the freshly inoculated culture. At higher concentrations of CH_3HgCl, bacterial growth began to be inhibited and $(CH_3)_2Hg$ evolution leveled off.

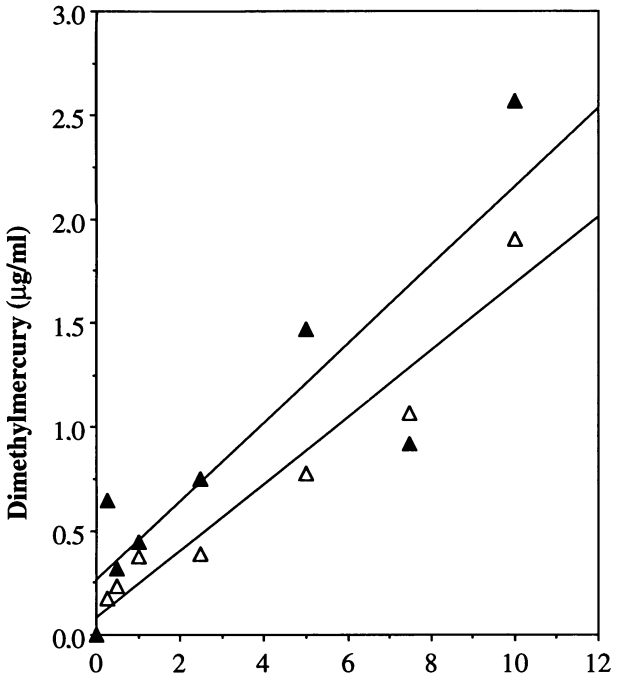

FIG. 2. Dimethylmercury production by different cultures of *D. desulfuricans* LS (△) and API (▲). Cultures were spiked with the different concentrations of CH_3HgCl at the start of the experiment 5 min after the inoculum was administered.

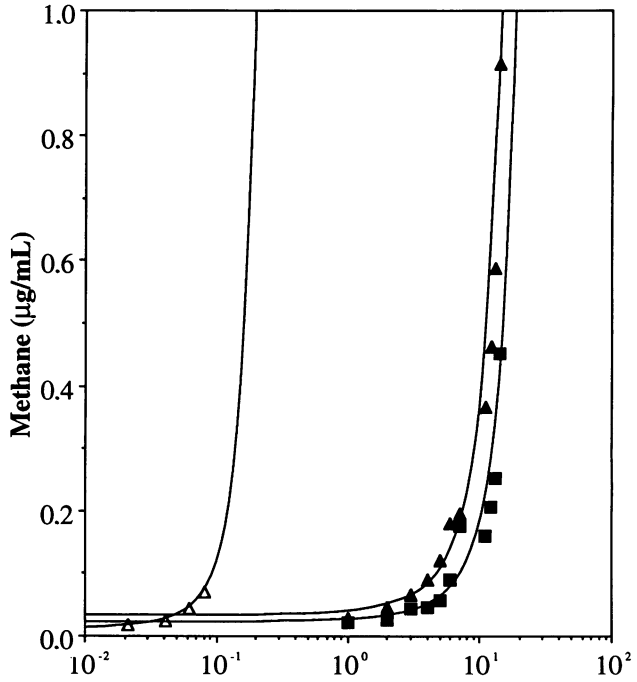

FIG. 3. Kinetics of methane formation in the headspace (8.3 ml) of serum bottles (50 ml) with *D. desulfuricans* LS in the presence of high sulfate concentrations (4.5 g · $liter^{-1}$) with a daily formation of $10^{0.10d}$ (▲) and low sulfate concentrations (0.090 g · $liter^{-1}$) with a daily formation of $10^{0.08d}$ (■). The experiments were performed by spiking cultures with 100 μg of CH_3HgCl ml^{-1} and incubating the cultures over a 15-day period. Methane evolution from the complete degradation of 1 μg of CH_3HgCl ml^{-1} by an active culture of *P. putida* FB-1 having a short evolution rate of $10^{10.1d}$ is also shown (△).

Analysis of headspace gas over *D. desulfuricans* cultures revealed the presence of methane, which increased significantly with sulfate content (Fig. 3). In control experiments, with the same amount of ethanol but without methylmercury, no methane was detected. The rate of methane formation from 100 μg of CH_3HgCl ml^{-1} added to *D. desulfuricans* cultures in exponential growth was 2 orders of magnitude less than that obtained from 1 μg of CH_3HgCl added to active cultures of *P. putida* FB-1.

Gaseous elemental Hg was not observed in the headspace of *D. desulfuricans* cultures with high and low sulfate contents, whereas traces of ionic mercury were found in the aqueous phase after toluene extraction (0.458 ± 0.21 mg · $liter^{-1}$ with high sulfate content and 0.365 ± 0.21 mg · $liter^{-1}$ with low sulfate content). No relationships were found between ionic Hg and other intermediates produced during CH_3HgCl decomposition.

The pH values measured in *D. desulfuricans* cultures after 14 days decreased from 7.5 (uninoculated samples) to a mean of 6.23 ± 0.03 because of H_2S production. At CH_3HgCl concentrations above 10 mg · $liter^{-1}$, the pH rose to neutral value, indicating growth inhibition, corroborated by a significant decrease in cells per liter. The methane detected in the headspaces was higher at low pH (Fig. 4) and decreased sharply to low content at pH values above 6.4.

To understand the degradation of methylmercury to various forms of Hg, a speciation study was performed in two separate 1-liter serum bottles, one amended with 100 mg of CH_3HgCl and the other amended with 100 mg of $HgCl_2$. The results of Hg speciation in the centrifuged solid material (pellet) from both experiments are reported in Table 1. The dry weights of *D. desulfuricans* LS cells contained in the pellets with CH_3HgCl and $HgCl_2$ were 55.8 and 46.7 mg · $liter^{-1}$, respectively. The cells spiked with CH_3HgCl contained only 0.058 mg of methylmercury $liter^{-1}$, corresponding to 0.91 μg · mg^{-1} (dry weight). A relatively high level of toluene-extractable Hg (0.38 mg · $liter^{-1}$ or 6.95 μg · mg^{-1}) was also found in the cell pellet spiked with $HgCl_2$, but purge-and-trap GC–Fourier transform infrared spectroscopy analysis showed that the content of toluene-extractable Hg did not correspond to methylmercury concentrations as in the previous sample. Dimethylmercury was not detected in the biomass of either sample.

The acid-insoluble fractions were analyzed by X-ray diffractometry. The materials were classified as metacinnabar (cubic HgS) from the spectrum and from the highest peak of relative intensity at 26°35′ theta. In the sample spiked with $HgCl_2$, 98% of the total Hg precipitated. The recovery by weight was 112.9 mg of HgS, corresponding to 97.4 mg · $liter^{-1}$ as total Hg. In a 1-liter culture spiked with 100 mg of CH_3HgCl, the gray precipitate contained only the 10.7 mg (dry weight) of Hg, partly as metacinnabar, $liter^{-1}$. The precipitate was not black or dense as in the experiment with $HgCl_2$ spikes. Previous studies (15–17) suggest that another insoluble species of Hg may be present in our CH_3HgCl-spiked cultures. In a separate experiment, when 10 mg of CH_3HgCl was added to 10 ml of *D. desulfuricans* culture, a

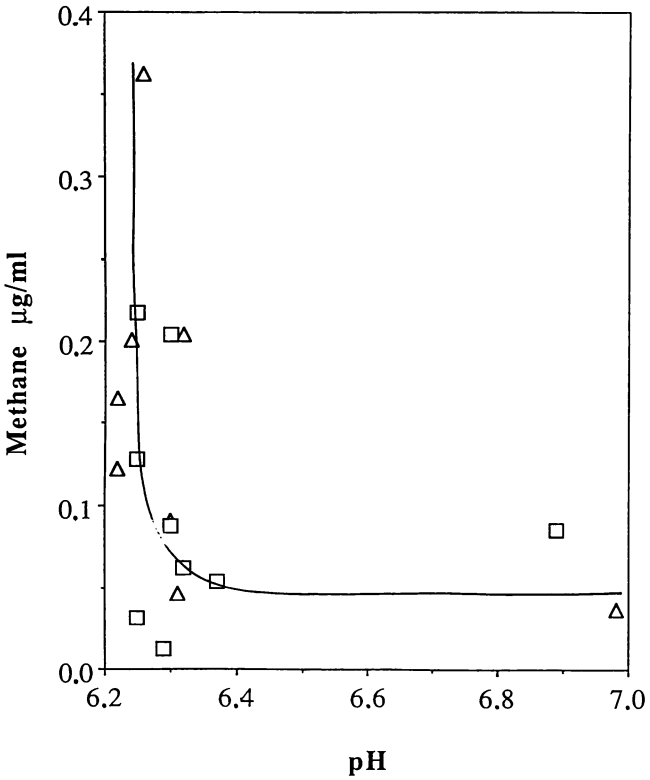

FIG. 4. Methane formation in the headspace by *D. desulfuricans* LS (△) and API (□) in relation to pH values determined in each sample after 15 days of incubation.

white precipitate formed immediately and no other chromatographic forms of Hg were detected (Fig. 5). This species was identified by GC in line with mass spectrometry to have a retention time of 11.3 min and a molecular weight of 464 and to contain Hg. No authentic standard was used, but the identification was based on the mass spectra from molecular fragmentation (mass/charge ratio [*m/z*]), which are shown in Fig. 5. The fragments were as follows: m/z = 47 for $[CH_3\text{-}S]^+$, m/z = 202 for $[Hg]^+$, m/z = 217 for $[CH_3\text{-}Hg]^+$, m/z = 249 for $[CH_3\text{-}Hg\text{-}S]^+$, m/z = 264 for $[CH_3\text{-}Hg\text{-}S\text{-}CH_3]^+$, and m/z = 464 for $[CH_3\text{-}Hg\text{-}S\text{-}Hg\text{-}CH_3]^+$. The mass/charge ratios of various peaks identified this species as dimethylmercury sulfide. This Hg species was observed to decompose quickly and visibly if exposed to air, turning from white to gray, and to form dimethylmercury, identified the next day by purge-and-trap GC–Fourier transform infrared spectroscopy.

TABLE 1. Hg speciation in the cell pellet of *D. desulfuricans* LS

Hg species	Hg concn [mg · liter^{-1} (µg · mg^{-1})] in[a]:	
	Sample I	Sample II
Toluene extractable organic	0.058 (0.91)	0.38 (6.95)
Methylmercury	0.058 (0.91)	ND
Dimethylmercury	ND	ND
Acid extractable inorganic	0.023 (0.36)	0.489 (9.19)
Insoluble (metacinnabar)	10.7	112.9[b] (97.4 mg · liter^{-1})

[a] Cultures were incubated at 28°C for 15 days and amended with 100 mg of methylmercury chloride ml^{-1} (sample I) or 100 mg of mercury chloride ml^{-1} (sample II). Values in parentheses are for cell dry weight. ND, not detected.
[b] Analyzed by weighing and qualitatively by X-ray diffractometry.

DISCUSSION

The methylmercury resistance in *D. desulfuricans* was due to the transformation of methylmercury to insoluble dimethylmercury sulfide, which reacted with H_2S from the dissimilative reduction of sulfate to give dimethylmercury, methane, and metacinnabar. Methylmercury resistance in anaerobic bacteria due to H_2S production has already been reported by Pan-Hu et al. (30), who isolated a strain (T-2) of *C. cochlearium* harboring methylmercury-decomposing activity in a plasmid. Mercuric reductase activity typical of Hg-resistant bacteria and the volatilization of Hg(0) were not detected in this strain. The ability of *C. cochlearium* to generate H_2S was harbored in the same plasmid as Hg-demethylating activity, although the demethylating mechanism was unknown (30). The results of our experiments suggest that Pan-Hu and Imura (30) observed methylmercury disappearance from *C. cochlearium* T-2 by a mechanism similar to that found by us in *D. desulfuricans*.

Chemical volatilization of methylmercury in the presence of H_2S was previously observed by Rowland et al. (32). Craig and Bartlett (15) first investigated this reaction in detail by nuclear magnetic resonance and mass spectrometry. The reaction of methylmercury with H_2S first produced a white compound identified as dimethylmercury sulfide. In time, this compound turned from white to black, and it was in this secondary reaction that the volatilization and loss of methylmercury occurred. In a further experiment with CH_3HgCl and H_2S, the white precipitate $[(CH_3Hg)_2S]$ formed, and after 3 days $(CH_3Hg)_2Hg$ was found in the vapor phase. However, methane was not detected in this experiment.

In the present biological experiments, the disappearance of CH_3HgCl was also attributed to $(CH_3Hg)_2S$ formation and its decomposition to $(CH_3)_2Hg$. The evolution of this organomercurial depended linearly on subtoxic concentrations of CH_3HgCl (10 µg · ml^{-1}), whereas at toxic concentrations, the inhibition of cell activity and subsequent H_2S production lowered methylmercury degradation to 5%. On the other hand, chemical experiments in our biological system showed methane production by *D. desulfuricans* cultures at both high and low concentrations of sulfates. The methane formation rate from methylmercury additions was low, being 2 orders of magnitude less than the methane production rate of *P. putida* FB1. The degradation of methylmercury followed by methane formation over a period of 15 days suggests that the degradation mechanism is different from that for broad-spectrum Hg-resistant bacteria (34).

Recently, Oremland et al. (27) found that methylmercury as $^{14}CH_3HgI$ added to freshwater estuarine sediments and to two anaerobic bacterial cultures of *Desulfovibrio gigas* and *Desulfovibrio africanus* was degraded over a period of days. The authors demonstrated that the anaerobic demethylation was due to oxidative demethylation followed by $^{14}CO_2$ and $^{14}CH_4$ volatilization. On the basis of time (3 weeks), this methane may also have arisen by the reaction of methylmercury with H_2S present in the anoxic environment. Moreover, Wood et al. (36) reported that dimethylmercury could be degraded under acid conditions to produce methane and monomethylmercury. The pHs determined in our *D. desulfuricans* cultures were acidic and were related to methane production in both strains.

The overall biological and chemical reactions are therefore as follows: $2CH_3Hg^+ + HS^- \rightarrow (CH_3Hg)_2S + H^+$, $(CH_3Hg)_2S \rightarrow (CH_3)_2Hg + HgS$, and $(CH_3)_2Hg + H^+ \rightarrow CH_4 + CH_3Hg^+$. The products are summarized as follows. (i) Dimethylmercury sulfide was produced instantly in the

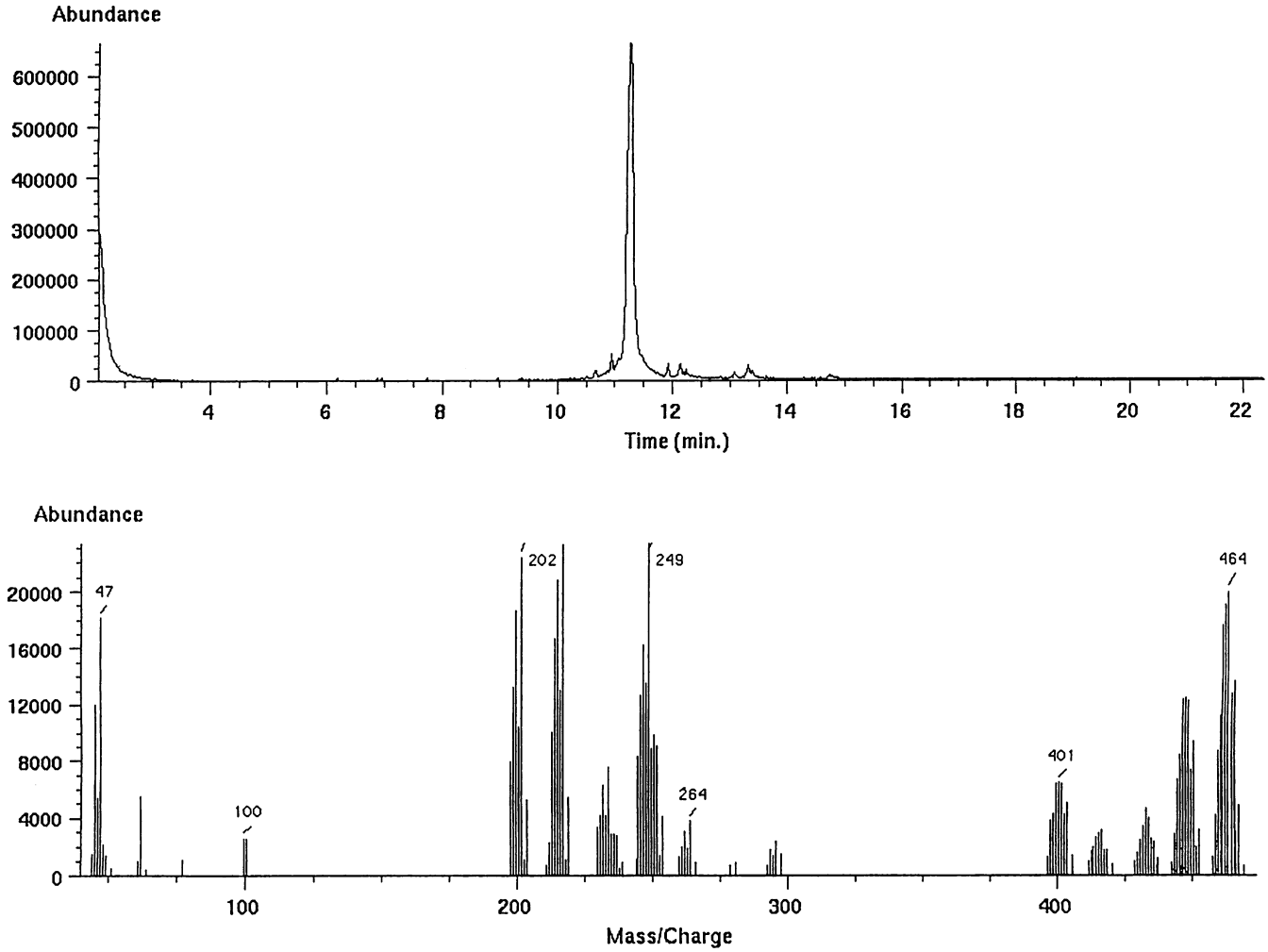

FIG. 5. Mass spectrum of dimethylmercury sulfide extracted from a culture of *D. desulfuricans* LS with chloroform. The relative retention time (top) and the abundance of molecular fragmentation (bottom) are shown.

reaction with H_2S from the biological sulfate reduction. The insolubility of this organomercurial conferred methylmercury resistance. Dimethylmercury sulfide was measured qualitatively, and a new method should be developed to detect it in the environment. (ii) Dimethylmercury formed slowly and exponentially in the liquid culture in a sulfate-rich medium. The low rate of formation was due to its secondary origin from $(CH_3Hg)_2S$ decomposition. (iii) The gray precipitate obtained from the pellet was probably a mixture of metacinnabar and dimethylmercury sulfide. However, HgS production is not the main product of methylmercury resistance in *D. desulfuricans* strains. (iv) Methane was probably produced by the degradation of dimethylmercury under the acidic conditions (pH 6.3) of cultures. This molecule is stable in aqueous solution above pH 10.5. (v) Methylmercury probably formed back from dimethylmercury decomposition. (vi) The traces of ionic Hg observed are difficult to correlate with intermediates, and we do not know yet whether it is a reaction product or is formed during the extraction of other Hg species.

The nonspecific mechanism of methylmercury resistance in *D. desulfuricans* consists of keeping intracellular concentrations of CH_3Hg^+ at subtoxic levels (0.9 $\mu g \cdot mg^{-1}$ [dry weight]) by continuous production of H_2S and then by rapid precipitation out of the cells as dimethylmercury sulfides, which later decompose to several by-products. Dimethylmercury was not detected in the cell pellet. The nonpolar molecule of dimethylmercury (boiling point = 98°C) presumably passively diffuses faster than elemental mercury (boiling point = 356.58°C) produced by Hg-resistant bacteria. The cytoplasmic pH is known to be higher than that of the medium outside *D. desulfuricans*, so the sulfate reduction system works sufficiently to reduce the CH_3HgCl content by producing H_2S. Outside the cell, the pH is lower and dimethylmercury has a better chance of being converted to monomethylmercury and methane.

An important point is that the strain *D. desulfuricans* LS has produced methylmercury from small additions of $HgCl_2$ (5, 11) and was recently used as a standard strain for ionic mercury conversion to methylmercury in an intercalibration exercise (28). Three laboratories detected a mean methylmercury value of 13.98 ± 5.3 $ng \cdot ml^{-1}$ in axenic cultures previously spiked with 5 μg of $HgCl_2$ ml^{-1} during a 2-day incubation. Moreover, sulfate-reducing bacteria were shown to be the best candidates for synthesizing methylmercury from inorganic mercury in the environment and under labo-

ratory conditions (11, 14, 22). The question is whether the sulfate-reducing bacteria produce methylmercury only or whether methylmercury is the most stable of several organic mercury species produced.

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