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Screening of Common Bacteria Capable of Demethylation of Methylmercuric Chloride

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Data gathered on the distribution of mercury in the environment, following the initial report of human poisonings in the Minamata Bay area of Japan, has shown the presence of inorganic mercury in aquatic systems to be of particular importance because of its subsequent conversion to the more toxic organic forms (EYL et al. 1970). While several studies have demonstrated mercury methylation by populations of bacteria, the purpose of this study was to screen forty common bacteria found in soils and sediments to determine their potential role in the cycling of mercurial species.

While microbial processes in the methylation of mercury have been well documented, (JENSON and JERNLOV 1969, VONK and SIJPESTEIJN 1973, and JERNLOV 1972) there is little definitive information on the degradation of organic mercurials to forms less toxic to microorganisms (LANDNER 1971). SPANGLER et al. (1973a) isolated 30 bacterial species and environmental samples which were capable of demethylating organic mercury. Two isolates were Gram positive cocci, two were Gram positive bacilli, with the remainder Gram negative bacilli having the characteristics of Pseudomonas (SPANGLER et al. 1973a, SPANGLER et al. 1973b). SCHOTTEL et al. (1974) found strains of Escherichia coli resistant to both phenylmercuric acetate and methylmercuric chloride, apparently as a result of enzymatic reduction of divalent mercury to the volatile Hg^{o}. BRUNKER and BOTT (1974) reported volatilization of mercury from a mercuric chloride solution by a yeast of the genus Cryptococcus. There have been, however, few bacterial genera reported which degrade organic mercurials to inorganic products.

MATERIALS AND METHODS

Selection of Microorganisms

Forty bacterial strains found in soils, sediments, and in sewage effluents were chosen to determine their ability to demethylate methylmercuric chloride. Ubiquitious microbes were selected to represent flora found in a broad range of environmental substrates. Eleven of the microorganisms were carried as stock cultures in the Department's microbiology laboratory; the remainder were obtained from the American Type Culture Collection (ATTC), Rockville, Maryland.

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Acclimation

Microorganisms were acclimated to methylmercuric chloride in sterile bubbler units at their optimum temperature in 200 ml of holding medium recommended for each organism by American Type Culture Collection (HATT and LESSEL 1974). Tolerance to organic mercury was achieved by daily addition of 0.25 mg/L (1 µM/L) methylmercuric chloride to a final concentration of 2.5 mg/L (10 µM/L). An adequate supply of nutrients was assured by daily addition of 1 ml of ten strength culture medium. Acclimation and screening tests were performed under anaerobic conditions for Clostridium perfringens, Clostridium bifermentans, Desulfovibrio desulfuricans, and Desulfovibrio aestuarii. The remaining microorganisms were cultured aerobically. Every other day, bacteria were subcultured in the appropriate holding media to assure continued growth of the cultures during exposure.

Screening Tests on Demethylation

Screening tests were performed under anaerobic conditions for strict anaerobes and aerobically for aerobes and facultative anaerobes. Eighteen-hour cultures of each organism, grown in holding media, were concentrated and washed so that approximately 50×10^6 cells were added to individual bubblers containing 300 ml of sediment. After 18 to 24 hr of incubation at ambient temperature, sterile methylmercuric chloride was added to produce a final concentration of 2.5 mg/L. Bubblers were prepared in duplicate.

Analysis of total mercury, methylmercury, and dialklmercurials in all test bubblers were made on 3-day intervals over a period of 16 days. Trap solutions were examined for the reaction products of volatile dialkylmercury compounds and mercuric bromide. Sterile blanks containing sediment and water served as controls to monitor background mercury and changes in mercury species by non-biological processes.

Selection and Characterization of Substrate

Clay sediment from a freshwater pond was selected as a culture medium to approximate nutrients existing in situ. The concentration of organic and inorganic nutrients was assayed (Total Organic Carbon 2,400 mg/L, Total Nitrogen 9.00 mg/L, Total Phosphorus 2.20 mg/L, pH 7.2) and preliminary trials using Pseudomonas aeruginosa in heat sterilized soil were conducted to confirm that the sediment had sufficient nutrients to adequately support microbial growth for the duration of the screening tests.

Experimental Apparatus

Sterilized bubblers (500-ml gas washing bottles) contained 300 ml of sediment and water, 50:50 by volume at pH 7. Humidified compressed air was passed through a membrane filter and bubbled into each flask to aerate the system and to flush any volatile mercury compounds produced by demethylation into a trapping solution (consisting of KBr - 100 g, $HgBr_2$ - 15 g, distilled water - 1000 ml) as described by SPANGLER et al. (1973a). Units containing anaerobic cultures were flushed with sterile nitrogen twice daily for one hr.

Chemical Analyses

Total Mercury Determination.--Total mercury was analyzed using the flameless cold vapor absorption method of UTHE et al. (1970) and HATCH and OTT (1968) using a Perkin-Elmer mercury analyzer (MAS-50). Analysis of total mercury in mud and sediment required a special digestion to oxidize all protein-mercury or sulfhydryl mercury bonds. The wet oxidation procedure recommended by EPA (1971) was used. The limits of detection of total mercury using this procedure was 0.2 µg/L mercury.

Determination of Alkylmercury Compounds.--A modification of the Westoo Method based on a two-step extraction of methylmercury and analysis by gas chromatography was employed (SWED. WATER AND AIR POLL. RES. LAB. 1970). Gas chromatograph conditions were: gas chromatograph; Micro-Tek MT-220 - with a 1.0 m glass column (4 mm ID) packed with 1.5% OV-17 and 1.5% OF-1 on Chromosorb W 80-100 mesh. Carrier gas was purified N_2 at 120 ml/min and an inlet temperature of 170°C. Column temperature was maintained at 145°C and the electron capture detector, (Tritium) at 165°C. The sensitivity of this method was 10 pg for methylmercury, 25 pg for ethylmercury and 200 pg for phenylmercury.

Determination of Dialkylmercury Compounds.--A combination of mercury analyzer and gas chromatograph, as recommended by LONGBOTTOM (1972), DRESSMAN (1972), and LONGBOTTOM and DRESSMAN (1973) was employed. Separation was accomplished using a gas chromatography column packed with 5% DC-200 and 3% OF-1 on gas chrom 80-100 mesh. Temperature programming was used to reduce analytical time. The separated compounds were subsequently combusted in a flame ionization detector from which the resulting free mercury was cooled and passed to the cold vapor mercury analyzer. The analytical detection limits in this procedure was 0.5 µg dialkylmercury.

RESULTS AND DISCUSSION

Of the 40 microorganisms tested, only 27 were able to tolerate methylmercuric chloride concentrations of 0.37 mg/L (1.5 µM/L) to 2.5 mg/L (10 µM/L). A list of microbes and their level of acclimation to organic mercury is given in Table 1.

Sixteen aerobes that showed growth in the cultures during acclimation to methylmercuric chloride were also positive for demethylation. Vibrio cuneatus, however, which showed growth during acclimation, was unable to demethylate the compound. Of the 4 anaerobes showing good tolerance to organic mercury, only Desulfovibrio desulfuricans showed demethylation under anaerobic conditions.

Demethylation of over 60% of the initial methylmercury concentration occurred within cultures of Enterobacter aerogenes, Serratia marcescens, Proteus mirabilis, Enterobacter cloacae, Providencia sp., Citrobacter freundii, and Pseudomonas fluorescens. Demethylation under aerobic conditions ranged from 20-84%. Desulfovibrio desulfuricans showed 32% demethylation during screening tests under

TABLE I

TOLERANCE OF SELECTED BACTERIA TO METHYLMERCURIC CHLORIDE

Microorganism	CH_3HgCl level attained (mg/L)
Achromobacter pestifer	2.5
Aeromonas hydrophila	-*
Aeromonas liquefaciens	-
Arthrobacter aurescens	-
Arthrobacter globiformis	-
Bacillus macerans	0.62 ‡
Bacillus megaterium	0.62
Bacillus subtilis	2.5
Brevibacterium fulvus	-
Brevibacterium linens	-
Brevibacterium sp.	-
Citrobacter intermedius	2.5
Citrobacter freundii	2.5
Closteridium bifermentans	2.5
Closteridium perfringens	2.5
Desulfovibrio aestuarii	2.5
Desulfovibrio desulfuricans	2.5
Enterobacter aerogenes	2.5
Enterobacter cloacae	2.5
Escherichia coli	-
Flavobacterium arborscens	2.5
Flavobacterium marinotypicum	0.62
Hyphomicrobium indicum	2.5
Paracolobacterum coliforme	2.5
Proteus mirabilis	2.5
Providencia sp.	2.5
Pseudomonas aeruginosa	2.5
Pseudomonas fluorescens	2.5
Pseudomonas fragi	-
Pseudomonas nigrifaciens	0.62
Pseudomonas stutzeri	-
Sarcina lutea	2.5
Serratia marcescens	2.5
Serratia plymuthica	2.5
Staphylococcus sp.	2.5
Vibrio albensis	2.5
Vibrio cuneatus	0.62
Vibrio cyclosites	-
Vibrio fisheri	-
Vibrio marinopraesens	

*Microorganisms could not tolerate as little as 0.37 mg/L.

‡Microorganisms could not tolerate CH_3HgCl concentration of more than 0.62 mg/L.

anaerobic conditions and was the only anaerobe with significant demethylating ability (see Table 2).

TABLE 2

DEMETHYLATION SCREENING TESTS FOR SELECTED BACTERIA AT AN INITIAL METHYLMERCURIC CHLORIDE CONCENTRATION OF 2.5 mg/L (10 μM/L)

Microorganism	% CH_3HgCl Reduction
Serratia marcescens	84
Providencia sp.	83
Pseudomonas fluroescens	79
Citrobacter freundii	71
Proteus mirabilis	71
Enterobacter aerogenes	69
Enterobacter cloacae	66
Paracolobacterum coliforme	59
Achromobacter pestifer	53
Serratia plymuthica	53
Staphylococcus sp.	53
Pseudomonas aeruginosa	52
Bacillus subtilis	47
Flavobacterium marinotypicum	37
Citrobacter intermedius	32
Desulfovibrio desulfuricans	32
Pseudomonas fragi	20
Vibrio cuneatus	1
Clostridium perfringens	negative
Clostridium bifermentans	negative
Desulfovibrio aestuarii	negative
Blanks:	
Anaerobic	9
Aerobic	7

Although all controls and test bubblers contained the same initial concentration of methylmercuric chloride (2.4 mg/L), loss of total mercury was higher in bubblers containing microorganisms than in blanks. Repeated determinations on methyl-, ethyl-, and phenylmercuric forms in the individual trapping solutions showed only slight loss of methylmercury chloride in the effluent gases (2.8 g/L H_3CHgCl vs 2.5 mg/L H_3CHgCl in cultures) with no measurable losses of the more volatile ethyl or phenyl forms. The same was true of the volatile dialkyl forms - dimethyl, diethyl, dipropyl, dibutyl and diphenyl mercury with no detectable levels found in either traps or culture flasks. The losses of total mercury of 5-43% which accompanied the disappearance of the parent methylmercury chloride compound probably represent reduction to the volatile Hg^o form which could not be quantified in SPANGLER'S (1973a) trapping solution. A number of authors (SUMMERS et al. 1972 and 1973; FURKAWA and TONOMURA 1972) have suggested formation of Hg^o in various pure cultures as have BISOGNI and LAWRENCE (1973) in the mixed cultures found in aerobic waste treatment systems. The loss of $HgCl_2$ from solution under strongly oxidizing treatment conditions (ORP +400 to +600 MV) in acid cleaned, fire polished glass, however, has also been documented by CARDNER (1977). Thus, there is some question as to the extent of the role of microorganisms in the reduction of the divalent compounds to the metal.

In summary, 21 bacteria were capable of degrading methylmercury chloride as measured by the disappearance of that species from the soil culture media. Disappearance of the methylated form was also accompanied by loss of total mercury in the culture media--probably as a result of reduction of the initial metabolite to volatile Hg^o. The role of bacteria in the reduction step is not clear at this time although several authors (SUMMERS et al. 1972 and 1973; BISOGNI and LAWRENCE 1973) have suggested facilitated reduction rates in the presence of microorganisms.

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