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Plasmid-Controlled Mercury Biotransformation by *Clostridium cochlearium* T-2

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A strain of *Clostridium cochlearium* having methylmercury-decomposing ability was isolated. The ability was cured by the treatment with acridine dye and recovered by the conjugation of the cured strain with the parent strain. The cured strain then showed the activity to methylate mercuric ion as previously reported (M. Yamada and K. Tonomura, J. Ferment. Technol. **50**:159-166, 1971). These results and the agarose gel electrophoretic pattern of the deoxyribonucleic acids from the lysates indicate a possible role of plasmids in controlling the mercury biotransformation of the two opposite directions in a single bacterial strain: methylation in the absence of the plasmid and demethylation in the presence of it. A possible mechanism for mercury resistance involving hydrogen sulfide is discussed.

According to the experimental results so far obtained, biotransformation of mercury compounds by microorganisms may proceed in two directions, which are contrary to each other. One is methylation of inorganic mercury, and the other is degradation of organic mercury, resulting in the formation of inorganic mercury. Each of these abilities has generally been considered to belong to different bacterial strains and to control the mercury cycle in the environment.

Of the various microorganisms, *Clostridium cochlearium* T-2 was reported to have a relatively high mercury-methylating activity (22), and methylcobalamin, a vitamin B_{12} analog, seems to be responsible for this methylation of mercury (8).

During cultivation for several generations of a preparation of *C. cochlearium* in the presence of methylmercury, we have recently found a strain of this anaerobe having abilities to decompose organic mercurials added to the medium and to generate hydrogen sulfide (14). By characterization of this organomercury-decomposing activity, we present evidence that biotransformation of mercury compounds towards opposite directions, methylation and demethylation, can be managed by a single bacterial strain, depending on the absence or presence of a transferable gene, probably a plasmid.

MATERIALS AND METHODS

Organism and culture. A lyophilized stock of *C. cochlearium* T-2 was kindly supplied by K. Tonomura of Osaka Prefectural University. The organism was routinely grown at 30°C under an atmosphere of nitrogen in a nutrient broth (pH 7) containing 7 g of meat extract, 10 g of peptone, 3 g of sodium chloride, 1 g of glucose, and 0.1 g of cysteine in 1 liter of the medium as described by Yamada and Tonomura (22).

To avoid confusion in distinguishing the strains of *C. cochlearium* T-2 used in this experiment, we tentatively named them as follows: T-2O is the originally supplied strain; T-2P, resistant to methylmercury (12.5 μg/ml), is the strain which is capable of decomposing organomercury and of generating hydrogen sulfide and obtained from T-2O by repeating more than 10 passages of culture in the presence of 1 μM methylmercury; T-2C is the cured strain from T-2P.

Curing test. Organisms (T-2P) were grown anaerobically in nutrient broth containing 400 μM acridine orange at 30°C. After 20 h, 0.1 ml of culture was plated on a thioglycolate medium (Difco Laboratories) containing 1.2% agar and incubated anaerobically in a GasPak jar (BBL Microbiology Systems) at 30°C for 20 h. Colonies were examined for methylmercury decomposing ability.

Examination of methylmercury decomposing activity. An overnight culture of the cells was diluted 50-fold with fresh medium containing 1 μM methylmercury and incubated at 30°C, and at certain time intervals 3 ml of the medium was withdrawn for determination of methylmercury, inorganic mercury, and total mercury.

The decomposing activity was determined by measuring the loss of methylmercury from the medium during incubation.

Transfer of demethylating activity by conjugation. The conjugation method is basically the same as described by Brefort et al. (1). Three colonies of mutants, resistant to rifampin, were obtained spontaneously by plating 5×10^8 cells of cured strain T-2C on a plate containing 30 μg of rifampin per ml, (frequency, 6×10^{-9}). The rifampin-resistant and demethylating activity-deficient strain (T-2C* MMC^- Rif^r) was used as a recipient.

The donor strain (T-2P MMC^+ Rif^s) and the recipient (T-2C*) were grown overnight separately in 50 ml of nutrient broth. A 0.1-ml amount of the donor (2.5×10^6 cells) and the recipient (2.5×10^6 cells) was

plated on GAM (Gifu anaerobic medium) agar medium (10 g of peptone, 3 g of soya peptone, 10 g of proteose peptone, 13.5 g of digested serum, 15 g of yeast extract, 2.2 g of beef extract, 1.2 g of liver extract, 3 g of glucose, 2.5 g of potassium dihydrogen phosphate, 3 g of sodium chloride, 5 g of soluble starch, 0.3 g of L-cysteine hydrochloride, 0.3 g of sodium thioglycolate, and 15 g of agar per liter; Nissui, Japan) and incubated anaerobically in a GasPak jar at 30°C. After 20 h, the bacteria were harvested by scraping with 1 ml of fresh nutrient broth, and 0.1 ml of the cell suspension obtained as above was plated on GAM agar medium containing rifampin (30 μg/ml), methylmercuric chloride (25 μg/ml), and ethylenediamine tetraacetic acid (EDTA) (1 mM), which was added for efficient selection based on mercury resistance. After 48 h of incubation, colonies on the selective agar medium mentioned above were examined for methylmercury decomposing activity and hydrogen sulfide-forming ability.

Agarose gel electrophoresis of plasmids. Microorganisms were grown overnight in 40 ml of nutrient broth and harvested by centrifugation. The plasmid-enriched fractions were prepared and subjected to agarose gel electrophoresis as described by Hansen and Olsen (5).

Examination of mercury methylating activity. The cured strain was anaerobically grown with 20 μM $HgCl_2$ in the absence or presence of vitamin B_{12} (10 μg/ml) in the dark as reported previously (6, 8). The methylmercury formed during 48 h of incubation at 30°C was determined.

Determination of total vitamin B_{12} and methylcobalamin. The supernatant obtained by centrifugation of sonicated *C. cochlearium* in 0.1 M acetate buffer (pH 4.6) at 10,000 × *g* for 15 min was employed for determination of total vitamin B_{12} (7). Methylcobalamin in the supernatant was isolated by paper chromatography before determination (7). *Lactobacillus leichmannii* ATCC-7830 was used for determination of the vitamin B_{12} analogs as reported previously (7).

Test for hydrogen sulfide production. Generation of hydrogen sulfide was detected by formation of heavy black precipitates in a triple sugar iron agar medium (TSI medium; Eiken, Japan) inoculated with bacteria as described by Lautrop et al. (10).

Resistance to mercury compounds. The level of mercury resistance (as expressed by the minimum inhibitory concentration, MIC) was determined in liquid cultures by the method of Clark et al. (2). The MIC is defined as the minimum concentration of mercurials which inhibits the increase of cell densities after 24 h of incubation.

Measurements. Methylmercury was measured by gas-liquid chromatography equipped with electron capture detector (6). Inorganic mercury was determined by the method of Magos (11). The total mercury was analyzed by atomic absorption spectrometry after the sample was digested with concentrated nitric acid as reported previously (8). The bacterial growth was measured by a spectrophotometer at 540 nm.

RESULTS

After 4 h of incubation with a strain of *C. cochlearium* (T-2P), methylmercury disappeared almost quantitatively from the culture medium (Fig. 1). However, this phenomenon was not observed when the organism was heated at 100°C for 10 min before inoculation. Accumulation of inorganic mercury in the medium increased along with the decrease of methylmercury, but total mercury still remained constant at this stage (Fig. 1). These results suggested that the linkage between mercury and carbon in methylmercury was split by the organism, resulting in the formation of inorganic mercury. No loss of mercury from the medium was observed during the incubation, and this indicated that any elemental mercury was not formed, although it was the final product in most of the cases of organomercury decomposition reported so far.

To discover whether the decomposing activity in this anaerobe was controlled by a plasmid as in the cases of other microorganisms such as *Pseudomonas* sp. (2), *Escherichia coli* (16), and *Staphylococcus* sp. (21), we made an attempt to correlate the loss of ability to decompose methylmercury with the removal of the plasmid. After 20 h of incubation with a subinhibitory concentration of acridine orange, the organisms were plated on agar medium and the colonies were tested for methylmercury-decomposing activity and hydrogen sulfide-producing ability. Of the colonies thus formed on the plate, 18 to 25% of the colonies lost their ability to decompose methylmercury (Fig. 2) and to produce hydrogen sulfide (data not shown). A band of deoxyribonucleic acid with lower mobility in the parent strain could be removed by curing with acridine dye (Fig. 3). These results suggest that the abil-

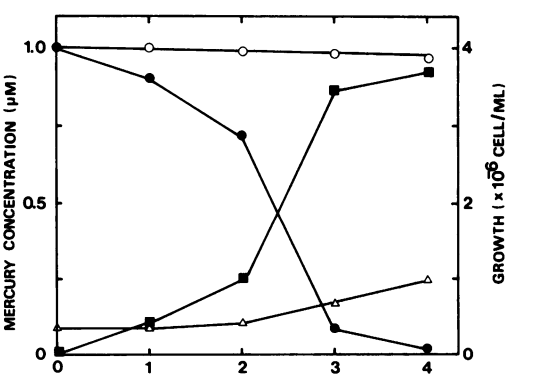

FIG. 1. *Decomposition of methylmercury and formation of inorganic mercury by C. cochlearium T-2P. C. cochlearium T-2P was grown anaerobically in nutrient broth containing 1 μM methylmercury at 30°C. Total mercury (○), methylmercury (●), and inorganic mercury (■) in the culture medium and the growth of C. cochlearium (△).*

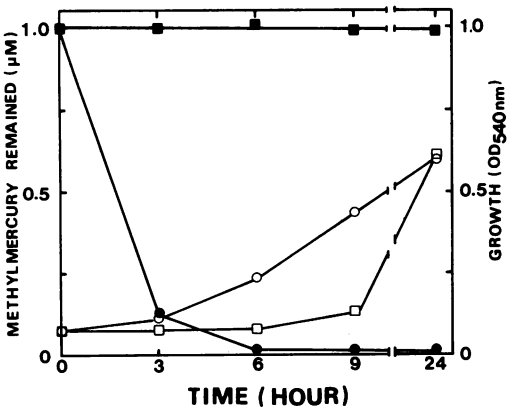

FIG. 2. *Decomposition of methylmercury by the parent strain (T-2P) and the cured strain (T-2C). The organisms were grown anaerobically in nutrient broth containing 1 μM methylmercury at 30°C. Decomposition of methylmercury by the parent strain (T-2P)* (●) *and the cured strain (T-2C)* (■). *Growth of the parent strain* (○) *and the cured strain* (□). *OD, Optical density.*

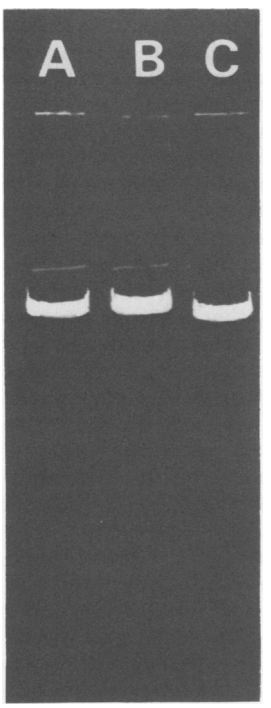

FIG. 3. *Agarose gel electrophoresis of DNAs from crude lysates. (A) lysate from conjugant; (B) lysate from parent strain (T-2P); (C) lysate from cured strain (T-2C).*

ity of the strain to decompose methylmercury is likely to be derived from a plasmid.

To provide further evidence that decomposing activity is plasmid mediated, we transferred the decomposing activity from the parent strain (T-2P) to the mutant cured strain (T-2C*). For efficient selection of the conjugant, we added EDTA to the medium containing methylmercury and rifampin because EDTA could raise the MIC of methylmercury for the parent strain about twofold. The ability to decompose methylmercury was transferred from the MMC^+ Rif^s donor to the MMC^- Rif^r recipient at a selection of Rif + MMC + EDTA at a frequency of 8.8×10^{-5}. The band of deoxyribonucleic acid which disappeared by the curing was recovered by the conjugation (Fig. 3). These results indicate that methylmercury-decomposing activity is mediated by a transferable gene, probably a plasmid. After the curing, the anaerobe became sensitive to mercurials (Table 1).

Next, the formation of methylmercury from inorganic mercury by the cured strain was examined. The results are summarized in Table 2. Determination of methylmercury by gas chromatography after 48 h of incubation with 20 μM $HgCl_2$ demonstrated that the cured strain has the ability to form methylmercury from inorganic mercury. And the activity was stimulated by the addition of 10 μg of vitamin B_{12} per ml to the culture medium. In contrast, the parent strain (T-2P) apparently remained inactive in methylating mercury. No significant difference in the content of total vitamin B_{12} and methylcobalamin was observed between the parent and the cured strain (Table 2).

DISCUSSION

Microbial methylation of mercuric ion (4, 8, 9, 13, 20, 22) and degradation of organic mercury (2, 3, 12, 15–18, 21) have been studied exten-

TABLE 1. *MICs of the parent strain and the cured strain*

Strain	MIC (μg/ml)	
	CH_3HgCl	$HgCl_2$
Parent (T-2P)	12.5	800
Cured (T-2C)	2.5	20

TABLE 2. *Mercury-methylating activity and cobalamin contents in the parent strain and the cured strain of C. cochlearium*

Strain	Methylmercury formed (ng/6 × 10^{10} cells per liter)		Co determined[a] (ng/mg of protein)	
	$-B_{12}$	$+B_{12}$	Total Co[a]	CH_3 Co
Parent (T-2P)	ND[b]	ND	1.70	0.47
Cured (T-2C)	960	1762	1.43	0.43

[a] Co, Cobalamin; CH_3 Co, methylcobalamin.
[b] ND, Not detected.

sively. From the experimental results reported so far, it appears that a certain strain of single species can methylate mercury and the other strain can decompose methylmercury. Of well-characterized bacterial species, however, no single bacterial strain has yet been reported to show both abilities to methylate mercuric ion and to decompose methylmercury.

In our recent experiment, a stock preparation of *C. cochlearium*, which had previously been able to methylate inorganic mercury (22), showed an ability to decompose methylmercury after repeated passages in the culture medium containing 1 μM methylmercury. This observation prompted us to recheck the microbiological properties of the strain having the methylmercury-decomposing ability. On the basis of morphological and physiological characteristics the strain was reconfirmed as a strain of *C. cochlearium* (18).

After curing with acridine orange, *C. cochlearium* T-2P lost their ability to decompose methylmercury (Fig. 2), and the deoxyribonucleic acid with lower mobility, probably a plasmid, was also eliminated from the strain (Fig. 3). However, the demethylating activity and the plasmid thus removed were recovered by the bacterial conjugation of the cured strain with the parent strain (Fig. 3). These experimental results likely suggest that the decomposing activity of this anaerobe is mediated, at least partly, by the plasmid. Recently, studies by Silver and his associates (2, 15, 21) on the mechanism of resistance to organomercurials in microorganisms have shown that some species were able to decompose organic mercury and that this ability was determined by a plasmid. Schottel (16) also reported that the plasmid-bearing strain of *E. coli* J531 (R831) could degrade organic mercury by an inducible hydrolase.

The formation of methylmercury from mercuric ion by the cured cells was further investigated. Interestingly, the cured strain showed the activity to methylate mercuric ion when incubated with $HgCl_2$ in the dark at 30°C for 48 h as reported previously (8). The methylmercury-forming activity of the cured strain was enhanced by the addition of vitamin B_{12} to the culture medium (Table 2) as observed by Yamada and Tonomura (22). It is notable that the contents of total vitamin B_{12} and methylcobalamin were not affected by the treatment with the acridine dye. In the culture of the parent cells (T-2P), any methylmercury could not be detected, despite the cellular methylcobalamin content, which was almost the same as that in the cured strain (T-2C) (Table 2). The methylating activity of the cured strain expressed by the amount of methylmercury formed per cell at the end of incubation after 48 h was 1.6×10^{-8} ng/cell. The methylating activity was too low to be detected at their early growth phase when the number of inoculated cells was the same as that in the culture for methylmercury decomposition.

On the other hand, degradation of methylmercury in the culture of the parent strain proceeded very rapidly even at the early growth phase. The decomposing activity of the parent strain was 2.5×10^{-4} ng/cell as expressed by the amount of methylmercury decomposed per cell at the end of 4 h of incubation. And, at this stage, accumulation of inorganic mercury was observed in the culture medium with a decrease of methylmercury (Fig. 1). In addition, the organism started to grow when the methylmercury was reduced to a limited level, whereas total mercury still remained at the original levels (Fig. 1). This observation may suggest that the remaining mercury had been converted to a less toxic form for the organism.

The ability to volatilize mercury from the culture medium seems to be generally required for acquiring plasmid-determined mercury resistance. But in the case of *C. cochlearium* T-2P, in which any mercury reductase activity was not detected, the mercury resistance may be due to the other mechanisms rather than to mercury volatilization. In the present experiment, the ability to generate hydrogen sulfide, shown by the parent strain of *C. cochlearium* (T-2P), was also cured by the treatment with the acridine orange (data not shown). This result suggested a possibility that the ability to generate hydrogen sulfide was also mediated by the plasmid. The cured strain showed a higher sensitivity to mercurials than did the parent strain (Table 1), suggesting that the hydrogen sulfide seems to be involved in the mercury resistance. The role of hydrogen sulfide in the expression of mercury resistance will be precisely discussed in our next paper.

To understand the reason why the original preparation of *C. cochlearium* T-2 showed the two opposite properties in the biotransformation of mercury compounds, we carried out a repeating passage experiment of the mixed culture of two strains of the anaerobe (*C. cochlearium* T-2P and T-2C; at the initial ratios of 1:9 and 1:99). The results shown in Fig. 4 revealed that the population of strain T-2P grew predominantly in both cases after several passages in the presence or absence of 1 μM methylmercury. This suggests the possibility that the original preparation of *C. cochlearium* (T-2O) was a mixture of the two strains, one of which had the

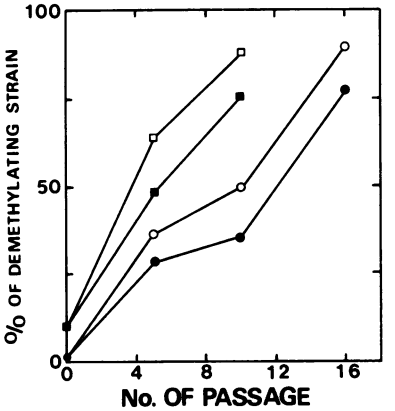

FIG. 4. *Change in population of two strains of C. cochlearium by repeating passages. Strain T-2P and T-2C were mixed at the initial ratios of 1:9 (■,□) and 1:99 (●,○). The mixture was grown anaerobically in nutrient broth in the absence (■,●) or presence (□,○) of 1 μM methylmercury for 24 h/passage. In both cases the inocula were adjusted to a level of about 10^5 cells/10 ml. After incubation, 10^5 cells were plated on the mercury-free GAM agar medium and incubated anaerobically in a GasPak jar at 30°C for 20 h. Fifty colonies were randomly picked and examined for methylmercury-decomposing activity.*

demethylating activity (T-2P) and the other (T-2C) did not.

The fact that a single bacterial strain can manage the mercury biotransformation toward the opposite directions, depending on the presence or absence of the plasmid, is of great interest when one considers the apparent equilibrium between organic and inorganic mercury compounds, the traffic of which is controlled by these numerous microorganisms in the environment.

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