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Role of Hydrogen Sulfide in Mercury Resistance Determined by Plasmid of *Clostridium cochlearium* T-2

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Abstract. Mercury resistance of *Clostridium cochlearium* T-2P was found to be controlled by a different mechanism from those reported so far since no mercury-reducing activity was detected in this strain.

The H_2S generating ability as well as the demethylating activity of this bacterium was eliminated by the treatment with acridine dye and recovered by the conjugation of the cured strain with the parent strain. In addition, the strain which lost their abilities to generate H_2S and to decompose methylmercury, showed higher sensitivity to mercurials than the parent strain. From these results, the genes conferring both the activities seemed to reside on the plasmid and the mechanism of mercury resistance was probably based on a detoxification mechanism involving methylmercury decomposition and inactivation of the inorganic mercury with H_2S.

Key words: Role of H_2S – Mercury resistance – Plasmid mediated – Methylmercury-decomposing activity – H_2S-forming ability – Inactivation of inorganic mercury – Bacterial conjugation – *Clostridium cochlearium*

The mechanism of plasmid-determined mercury resistance has been reported in many species of bacteria (Smith 1967; Novick and Roth 1968; Summers and Silver 1972; Kondo et al. 1974; Schottel et al. 1974; Summers and Sugarman 1974; Clark et al. 1977; Izaki 1977; Weiss et al. 1977; Olson et al. 1979). The biochemical basis of resistance are due to enzymatic degradation of organomercurials and reduction of inorganic mercury ion to elemental mercury which subsequently volatilized from the culture medium (Komura and Izaki 1971; Nelson et al. 1973; Schottel et al. 1974; Summers and Silver 1972; Tezuka and Tonomura 1976; Clark et al. 1977; Weiss et al. 1977; Schottel 1978).

Recently we found that the biotransformation of mercury compounds by a strain of *Clostridium cochlearium* T-2 toward the opposite directions, methylation and demethylation, could be managed by a single strain depending on the absence or presence of the transferrable plasmid (Pan-Hou et al. 1980).

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Abbreviations: MMC = methylmercuric chloride; Rif^s = sensitive to rifampin; Rif^r = resistant to rifampin; MMC^+ = methylmercury-decomposing activity plus; MMC^- = methylmercury decomposing activity minus

The plasmid of *C. cochlearium* T-2P, the strain having methylmercury decomposing activity, seemed to determine mercury resistance beside the decomposing activity, but mercury-reducing activity which has generally been considered to play an important role in the mercury resistance, was not detected in this strain. Thus, the biochemical basis for mercury resistance of this strain remained to be elucidated. During the characterization of the strain cured by acridine dye, we found that the hydrogen sulfide forming ability was also eliminated by the curing along with the plasmid DNA. This suggested that hydrogen sulfide might play an important role in the expression of mercury resistance of *C. cochlearium* T-2P (Pan-Hou et al. 1980).

We present in this paper convincing evidence that two genetic factors conferring the methylmercury-decomposing activity and hydrogen sulfide-forming ability, which are probably located on the same plasmid, determine mercury resistance by virtue of these activities.

Materials and Methods

Organism and Medium

Clostridium cochlearium T-2P carrying a mercury-resistant plasmid which confers the activities to decompose methylmercury and to generate hydrogen sulfide, was used as parent strain. The mercury sensitive cured strain T-2C, the rifampin resistant mutant strain T-2C* from the cured strain and the medium used in this study were the same as those previously described (Pan-Hou et al. 1980).

Preparation of Cell Suspension

The organisms grown in the presence of 1 μM CH_3HgCl were harvested by centrifugation at their late exponential growth phase and washed twice with 20 ml of Tris-HCl buffer (pH 7.5). The washed cells were suspended in the same buffer (1 g cells/10 ml) and employed as a cell suspension.

Determination of Susceptibility to Metal Compound

The level of metal resistance was determined in liquid cultures according to the method of Clark et al. (1977). Minimum inhibitory concentration is defined as the minimum concentration of metal compound which inhibits increase of cell densities after 24 h incubation.

Assay for Methylmercury Decomposition

The demethylating activity in the cell suspension was assayed in the following reaction mixture; 50 mM Tris-HCl buffer (pH 7.5), 10 mM

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glucose, 1 mM sodium thioglycolate, 1 μM [^{3}H]-CH_3HgCl (sp. act. 105 mCi/mmol) or [^{203}Hg]-CH_3HgCl (sp. act. 4 mCi/mg Hg) and cell suspension (final concentration 0.8 mg/ml). Aliquots (50 μl) of the reaction mixture were withdrawn and put on the paper disk at the time intervals. After drying, the radioactivity on the disk was measured by a liquid scintillation spectrometer (Aloka, Japan) for [^{3}H]-CH_3HgCl and by an Auto Well gamma system (Aloka, Japan) for [^{203}Hg]-CH_3HgCl. The decomposing activity was measured by following the loss of radioactivity from the reaction mixture during the incubation at 30°C. The reduced mercury (Hg°) treated in the same way was vaporized from the paper disk.

Test for Hydrogen Sulfide Production

Generation of hydrogen sulfide was detected by formation of heavy black precipitates in a Triple Sugar Iron agar medium (Eiken, Japan) as described by Lautrop et al. (1971).

Hydrogen sulfide forming ability was assayed by formation of methylene blue in the oxidation of hydrogen sulfide by dimethyl-p-phenylenediamine sulfate and a ferric salt as described by Lorant (1929). The organisms were grown anaerobically in 50 ml of nutrient broth at 30°C. The culture chambers were connected with two traps by glass tubing. Each trap consisted of 10 ml of a solution [0.5% $ZnSO_4$, 0.6% NaOH, 7% $(NH_4)_2SO_4$ and 5% glycerol] to absorb hydrogen sulfide. At the time indicated the cultures were bubbled for 15 min with purified nitrogen gas. The solutions in two traps were combined and hydrogen sulfide fixed by $ZnSO_4$ was determined as methylene blue by adding 2 ml of 0.2% dimethyl-p-phenylenediamine sulfate in 10 N H_2SO_4 and 1 ml of 0.5% $FeCl_3$. Methylene blue formed was determined by measuring absorption at 680 nm.

Transfer of Plasmid

C. cochlearium T-2P (MMC^+, H_2S^+, Rif^s) and the cured strain (MMC^-, H_2S^-, Rif^r) were used as donor and recipient, respectively. The method for plasmid transfer applied in the present experiment was as described previously except for using thioglycolate agar medium (Difco) instead of GAM agar medium (Pan-Hou et al. 1980). Colonies on the selective medium were checked for methylmercury-decomposing activity and hydrogen sulfide-forming ability.

Binding Experiment

Binding of [^{203}Hg]-$HgCl_2$ to the cells was measured in the reaction mixture containing 50 mM Tris-HCl buffer (pH 7.5), 10 mM glucose, 1 mM sodium thioglycolate, 1 μM [^{203}Hg]-$HgCl_2$ (sp. act. 1 mCi/mg Hg) and 0.8 mg/ml of cells. After incubation at 30°C, aliquots (0.5 ml) of the reaction mixture were removed and filtered on the membrane filters (Milipore Crop, pore size 0.3 μm) and the filters were washed twice with 3 ml of Tris-HCl buffer (pH 7.5). The radioactivity on the paper was counted by Auto Well gamma system.

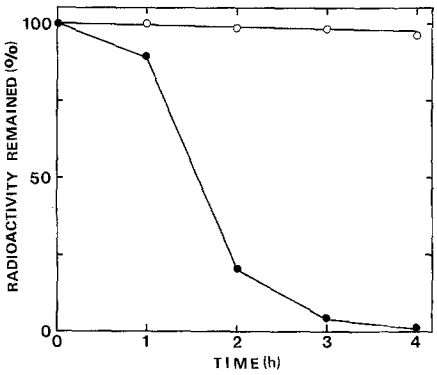

Fig. 1. Decomposition of radioactive methylmercury by *Clostridium cochlearium* T-2P. Assay conditions are as described in Materials and Methods. Symbols; [^{3}H]-CH_3HgCl (●), [^{203}Hg]-CH_3HgCl (○)

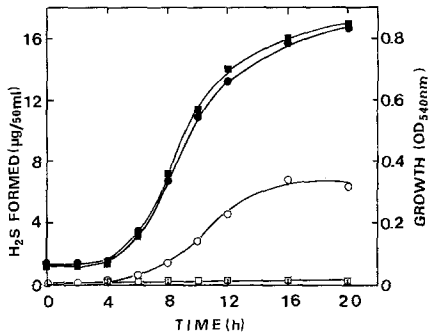

Fig. 2. Formation of hydrogen sulfide by the parent strain and the cured strain of *Clostridium cochlearium*. Formation of H_2S by the parent strain (T-2P) (○) and the cured strain (T-2C) (□) was measured according to the method indicated in the text. Growth of the parent strain (T-2P) (●) and the cured strain (T-2C) (■) was monitored by spectrophotometer at 540 nm

Table 1. Pattern of metal resistance of the parent strain and the cured strain of *Clostridium cochlearium*

Strain	Minimum inhibitory concentration (mM)					
	Cd^{2+}	Hg^{2+}	Cu^{2+}	Pb^{2+}	Zn^{2+}	Cr^{3+}
Parent T-2P	1.67	2.95	6.67	13.33	106.67	3.33
Cured T-2C	0.11	0.07	0.21	6.67	13.67	3.33

Results

When the cells (*Clostridium cochlearium* T-2P) were incubated in the presence of 1 μM [^{3}H]-CH_3HgCl, the radioactivity in the reaction mixture disappeared very rapidly. However, no detectable loss of the radioactivity of ^{203}Hg was observed when [^{203}Hg]-CH_3HgCl was used instead [^{3}H]-CH_3HgCl (Fig. 1). These observations suggested that the linkage between mercury and carbon in methylmercury was cleaved resulting in formation of inorganic mercury but not of elemental mercury.

The hydrogen sulfide-forming ability as well as the methylmercury-decomposing activity of the organism was eliminated from the parent strain by the treatment with acridine orange (Fig. 2). This result suggests that the ability to form hydrogen sulfide is correlated with the presence of the plasmid. To provide further evidence that the methylmercury-decomposing activity and the hydrogen sulfide-forming ability are associated with mercury resistance in this bacterium, transfer of these abilities were tried by bacteria conjugation. The abilities of methylmercury decomposition and hydrogen sulfide production were transferred from the donor strain (T-2P) to the recipient strain (T-2C*) at a frequency of 9.5×10^{-5}/donor cell and the conjugant showed similar mercury resistance as that of parent strain (T-2P).

The cured strain (T-2C) showed higher sensitivity than the parent strain (T-2P) to Cd^{2+}, Cu^{2+}, Zn^{2+}, and Hg^{2+} which have generally been considered to be reactive with hydrogen sulfide (Table 1).

No significant difference in the binding of the mercury was observed between the cured strain (T-2C) and the parent strain (T-2P) as shown in Fig. 3.

Discussion

We have recently reported that the conjugative plasmid of *Clostridium cochlearium* T-2P which confers the abilities to decompose methylmercury and to form hydrogen sulfide was able to be eliminated by the treatment with acridine dye and the cured strain (T-2C) became sensitive to mercury compounds compared with its parent strain (Pan-Hou et al. 1980). These observations suggested that mercury resistance of this bacterium might be mediated by the plasmid.

The volatilization of mercurials has been considered to be a general mechanism of mercury resistance as reported so far. A difference in the binding or uptake of the mercurials between the resistant and sensitive strain have also been put forward as one of the mechanism of mercury resistance (Greenaway 1972; Kondo et al. 1974). In the case of *C. cochlearium* T-2P, the experimental results suggest that the mercury resistance of this bacterium is ascribed to neither the enzymatic volatilization of mercury (Fig. 1) nor the depression of mercury binding or uptake (Fig. 3).

After curing with acridine orange, the organism lost its ability to form hydrogen sulfide (Fig. 2). However, hydrogen sulfide-forming ability thus removed, was recovered by the conjugation of the cured strain with the parent strain at a frequency of 9.5×10^{-5}/donor cell. In addition, the hydrogen sulfide-forming ability in the conjugant, which was resistant to mercury and rifampin, appeared to be derived from the same plasmid as that for the demethylating activity, since they were always transmitted together. No segregation of demethylating activity and hydrogen sulfide-forming ability was observed among the conjugant formed on the selective agar medium. From these results the genes conferring both activities seem to reside on the plasmid harbored by the parent strain (T-2P).

Plasmid mediated formation of hydrogen sulfide has been reported in many microorganisms (Layne et al. 1971; Ørskov and Ørskov 1973; Burkardt et al. 1978; Briaux et al. 1979). By use of the electron microscopy, Ashida and his coworkers reported that precipitation of cupric sulfide was found in the copper resistant cells, *Saccharomyces ellipsoideus*, and speculated that hydrogen sulfide might be required for acquiring the copper resistance (Ashida et al. 1963; Ashida 1965).

The minimum inhibitory concentration of Cd^{2+}, Zn^{2+}, Cu^{2+}, and Hg^{2+} for the parent strain of *C. cochlearium* were significantly higher than those for the cured strain T-2C (Table 1). These metal ions have been considered to be very reactive with hydrogen sulfide to form insoluble metal sulfides. These experimental results appeared to prove that hydrogen sulfide, by virtue of its reactivity to inactivate mercuric mercury, might play an important role in the expression of mercury resistance. Because the reaction product of organomercury decomposition in the mercury resistant strain is inorganic mercury which is still considered to be toxic for the organism and no mercury-reducing activity to form volatile elemental mercury from inorganic mercury has yet been detected in this bacterium.

The results presented above lead us to conclude that in the parent strain of *C. cochlearium*, resistant to mercury compounds, enzymatic degradation of organomercury to inorganic mercury and subsequent inactivation of the inorganic mercury with hydrogen sulfide to an insoluble mercuric sulfide, confer the bacterium resistance to mercury compounds. To our knowledges this is the first case on the plasmid-mediated mercury resistance that involves organomercury decomposition and hydrogen sulfide formation.

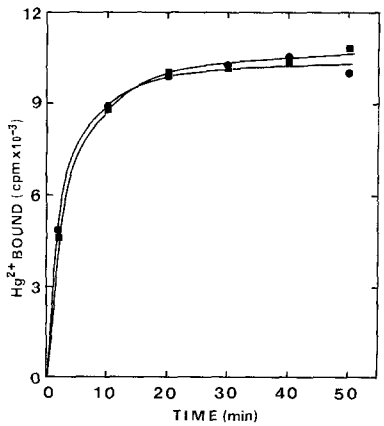

Fig. 3. Binding (or uptake) of mercuric chloride by the parent strain and the cured strain of *Clostridium cochlearium*. Symbols; Parent strain (T-2P) (●), Cured strain (T-2C) (■)

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References

Ashida J (1965) Adaptation of fungi to metal toxicants. Ann Rev Phytopathol 3:153–174

Ashida J, Higashi N, Kikuchi T (1963) An electron-microscopic study on copper precipitation by copper-resistant yeast cells. Protoplasma 57:27–32

Briaux S, Gerbaud G, Jaffe-Brachet A (1979) Studies of a plasmid coding for tetracycline resistance and hydrogen sulfide production incompatible with the prophage Pl. Molec Gen Genet 170:319–325

Burkardt H, Mattes R, Schmid K, Schmitt R (1978) Properties of two conjugative plasmid mediating tetracyaline resistance, Raffinose catabolism and hydrogen sulfide production in *Escherichia coli*. Molec Gen Genet 166:75–84

Clark DK, Weiss AA, Silver S (1977) Mercury and organomercurial resistance determined by plasmid in *Pseudomonas*. J Bacteriol 132:186–196

Greenaway W (1972) Permeability of phenyl-Hg^{+}-resistant and phenyl-Hg^{+}-susceptible isolates of *Pyrenophore avenae* to the phenyl-Hg^{+} ion. J Gen Microbiol 73:251–255

Izaki K (1977) Enzymatic reduction of mercurous ions in *Escherichia coli* bearing R factor. J Bacteriol 131:696–698

Komura I, Izaki K (1971) Mechanism of mercuric chloride resistance in microorganisms. I. Vaporization of a mercury compound from mercuric chloride by multiple drug resistance strain of *Escherichia coli*. J Biochem 70:885–893

Kondo I, Ishikawa T, Nakahara H (1974) Mercury and cadmium resistance mediated by the penicillinase plasmid in *Staphylococcus aureus*. J Bacteriol 117:1–7

Layne P, Hu ASL, Balows A, Davis BR (1971) Extrachromosomal nature of hydrogen sulfide production in *Escherichia coli*. J Bacteriol 106:1020–1030

Lautrop H, Ørskov I, Gaarsleu K (1971) Hydrogen sulfide producing variants of *Escherichia coli*. Acta Path Microbiol Scand Sec B 79:641–650

St Lorant I (1929) Über eine neue colorimetrische Mikromethode zur Bestimmung des Schwefels in Sulfiden, Sulfaten usw. Z Physiol Chem 185:245–266

Novick RP, Roth C (1968) Plasmid-linked resistance to inorganic salts in *Staphylococcus aureus*. J Bacteriol 95:1335–1342

Nelsen JD, Blair W, Brinckman FE, Colwell RR, Iverson WP (1973) Biodegradation of phenylmercuric acetate by mercuric resistant bacteria. Appl Microbiol 26:321–326

Ørskov I, Ørskov F (1973) Plasmid-determined H_2S character in *Escherichia coli* and its relation to plasmid-carried raffinose fermentation and tetracycline resistance characters. J Gen Microbiol 77:487–499

Olsen BH, Barkay T, Colwell RR (1979) Role of plasmids in mercury transformation by bacteria isolated from the aquatic environment. Appl Environ Microbiol 38:478–485

Pan-Hou HSK, Hosono M, Imura N (1980) Plasmid-controlled mercury biotransformation by *Clostridium cochlearium* T-2. Appl Environ Mircobiol 40:1007–1011

Schottel J, Mandal A, Clark D, Silver S (1974) Volatilization of mercury and organomercurials determined by inducible R-factor in enteric bacteria. Nature 251:335–337

Schottel JL (1978) The mercuric and organomercurial detoxifying enzymes from a plasmid-bearing strain of *Escherichia coli*. J Biol Chem 253:4341–4349

Smith DH (1967) R-factors mediate resistance to mercury, nickel, and cobalt. Science 156:1114–1116

Summers AO, Sugarman LI (1974) Cell-free mercury (II)-reducing activity in a plasmid-bearing strain of *Escherichia coli*. J Bacteriol 119:243–249

Summers AO, Silver S (1972) Mercury resistance in a plasmid-bearing strain of *Escherichia coli*. J Bacteriol 112:1228–1236

Tezuka T, Tonomura K (1976) Purification and properties of an enzyme catalyzing the splitting of carbon-mercury linkages from mercury-resistant *Pseudomonas* K-62 strain. J Biochem 80:79–87

Weiss AA, Murphy SD, Silver S (1977) Mercury and organomercurial resistance determined by plasmid in *Staphylococcus aureus*. J Bacteriol 132:197–208

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