Studies on Heavy-Metal Resistance in Clinical Isolates of *Proteus mirabilis*

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INTRODUCTION

The resistance of many microorganisms to metallic ions is associated with plasmid[1~6], such as the penicillinase plasmid in *Staphylococcus aureus* which determines resistance to mercury, cadmium, arsenate, lead, bismas and zinc[7] or R plasmid mediating resistance to mercury, cobalt and nickel which have been observed in *Escherichia coli*[8]. Although mediated by the same penicillinase plasmid[9], resistance to mercury and cadmium are controlled by quite different mechanisms. That the enzymatic transformation of inorganic mercury (Hg^{2+}) to metallic mercury (Hg^0), which is volatilized from growth media[1,10~14], is the cause of plasmid-mediated resistance to mercury in bacteria is generally acknowledged. The ability of various *E. coli*, *S. aureus* and *Pseudomonas aeruginosa* strains harboring different antibiotic-resistant plasmids to reduce inorganic mercury to metallic mercury has been demonstrated in a series of elegant studies by Summers et al.[15] and Schottel et al.[16,17]. Many authors have described resistance to mercury from the point of view morphology[18], genetics[1,10,12~14] and epidemiology[19~21].

It is noteworthy that the same plasmids which determine resistance to antibiotics also mediate resistance to metals. For example, the causes of "Minamata disease"[22] and "Itai-Itai disease"[23] in Japan are found to be methylmercury and cadmium, respectively. The role of R plasmids in drug-resistant bacteria has been widely studied and factors outside of the chromosomes are a major cause of the increase in such bacteria[14,21]. Establishment of a relationship between metal- and drug-resistant bacteria has been attempted in the hospital environment[25~27], but the factors for selecting metal resistance have yet to be identified. Heavy-metal-resistant microorganisms do not arise by chance, but, there must be selectional factors beyond mere drug resistance. One such selectional factor may be enviromental pollution by these metals[28].

With this in mind, we previously isolated strains of *S. aureus, E. coli*, *Klebsiella pneumoniae* and *P. aeruginosa* from hospital patients and investigated the distribution of their resistance to several metallic ions such as mercury, cadmium, arsenate and lead[29]. R plasmids carrying mercury resistance were demonstrated in 89.9% of the 198 Hg-resistant strains of *E. coli*[30] and 91.5% of the 94 Hg-resistant strains of *K. pneumoniae*[31]. We have previously described that *P. mirabilis* carrying Hg^{2+} resistant R plasmid (NR1) have volatilization activity of $^{203}Hg^{2+}$[11].

We have conducted our investigation of the metal and drug resistance using clinical strains of *P. mirabilis* isolated from Jikei University Hospital since 1975. We tested their resistance patterns and investigated the presence of R plasmids. Using radioactive $^{203}Hg^{2+}$, we assayed the volatilization of inorganic mercury with strains containing these mercury-resistant plasmids. This paper deals with the results of our 8-year survey of 417 *P. mirabilis* strains isolated from 1975 to 1982.

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MATERIALS AND METHODS

Bacterial strains A total of 417 strains of *P. mirabilis* were isolated from Jikei University Hospital from 1975 to 1982 and examined for susceptibility to three metals and four antibiotics. Control strains used in this study were *E. coli* K12-CSH2(R6) and *E. coli* K12-ML1410. *E. coli* K12-CSH2(R6) which carries R plasmid and it resistant to streptomycin (SM), tetracycline (TC), chloramphenicol (CP), Kanamycin (KM) and mercuric chloride (Hg^{2+}), was employed as the control for resistant culture, while *E. coli* K12-ML1410 was used as the sensitive culture. Using *E. coli* K12-ML1410-Nx and *E. coli* JE17 as recipients, we examined the conjugal transferability[30].

Antibiotics and chemicals For Hg, Cd and As, we used the metallic ions $HgCl_2$, $CdCl_2$ and Na_2HAsO_4, respectively. These metals were all dissolved in distilled water, sterilized by means of a membrane filter (Millipore Corp., pore size 0.45 μm), and added to agar medium in disignated concentrations. The agar dilution method was employed to determine minimal inhibitory concentrations (MIC) of the six antibiotics tested against *P. mirabilis* strains. Antibiotics used were SM, TC, CP and KM. Those cultures not inhibited by 25μg of SM, TC, CP or KM per m*l* were considered resistant to each of the antibiotics.

Media Heart infusion agar (Difco) was used for each antibiotic resistance test, and nutrient agar (Difco) was used for each metal resistance test.

Experiments of conjugation Clinical isolates of Hg-resistant *P. mirabilis* were cultivated for transfer for 5h in nutrient broth (Difco) with shaking. One milliliter of a culture containing about 10^6 of bacteria was mixed with an equal portion of the recipient culture similarly prepared. The mixture was statically incubated for 3-5h before plating about 0.01m*l* on a nutrient agar containing 25 μg of nalidixic acid (Nx) or refampicin (Rif) per m*l* plus 10 μg of $HgCl_2$ per m*l*. After overnight incubation at 37°C, the plates were compared with control plates containing individual drugs and the parent stains, as well as with Nx or Rif plus Hg or single antibiotic plates.

Radioactive mercury volatilization assays Radioactive mercury volatilization assays were conducted as described by Schottel et al.[16,17]. Cultures prepared overnight were diluted 1/100 into fresh media, grown to mid-log phase, and induced with Hg^{2+}. After 60 minutes, cells were either reinduced or harvested by centrifugation, washed by centrifugation with 50mM sodium phosphate buffer (pH6.8), and then suspended in phosphate buffer at a constant turbidity as determined by a klett colorimeter (equivalent to 2.75mg [dry weight]/m*l*). The cells then experienced a 10-fold dilution into an assay mixture of 50mM sodium phosphate buffer (pH6.8), 0.5mM ethylenediamine-tetraacetic acid, 0.2mM magnesium acetate, and 1.0mM β-mercaptonethanol. 2 or 5 μM $^{203}Hg^{2+}$ was added for mercury volatilization assays. Samples (20μl) were taken periodically and counted in a Packard liquid scintillation spectrometer.

RESULTS

Distribution pattern and the frequency of metal resistance in P. mirabilis The distribution curves of Hg-, Cd-, As- and Pb-resistance of 417 strains of *P. mirabilis* obtained by the agar dilution method show a clear-cut bimodal distribution of susceptibility to three of the metals and a single-peak resistance to Pb. Similar results were obtained in *E. coli*, *K. pneumoniae* and *P. aeruginosa*[28~30]. Resistance was demonstrated in media containing the following concentrations of the metals (μg/m*l*) : $HgCl_2$, 10; $CdCl_2$, 400; Na_2HAsO_4, 400. The resistance concentrations of these metals in agar were the same as that of *E. coli*, *K. pneumoniae* and *P. aeruginosa*[28].

As shown in Table 1, the frequency of resistance to these concentrations of Hg, Cd and As totaled 28.8, 95.7 and 57.1, respectively. As to resistance to antibiotics, they were most frequently resistant to SM (50.1%), followed by TC (41.7%), CP (32.1%) and KM (29.5%). The frequency of mercury resistance was 58.3% during the period from 1975 to 1977, but decreased to 16.8% from 1979 to 1982, as shown in Table 1. This decrease in frequency of resistance to Hg^{2+} exhibited in the *P. mirabilis* isolates also occurred in *E. coli*, *K. pneumoniae* and *S. aureus*[19]. Frequencies of resistance to Cd and As remained fairly constant throughout the study period.

Transfer of plasmids carrying Hg resistance As shown in Table 2, the whole complex pattern of

resistance carried over from the JMP121, JMP140, JMP197, JMP251 and JMP290 *P. mirabilis* strains to *E. coli* K12-ML1410-Nx at a frequency of about 10^{-3}.

Next, the resistance of these exconjugants was tested for transferability, using *E. coli* JE17-Rif as a recipient. The resulting exconjugants of *E. coli* K12-R^+ were then selected on nutrient agar with 100μg/m*l* of Rif together with a single selected drug or mercury in the same proportions mentioned above. After 3 or more hours of mating, it was noted that the total resistance pattern transfered at a high frequency to the *E. coli* JE17. Data on this second cycle are included in Table 2. This pattern of resistance continued to transfer well between the strains described in further cycles of testing.

Genetic properties of the R plasmids with Hg resistance As shown in Table 3, which gives the resistance patterns of mercury-resistant *P. mirabilis* strains, quadruple drug resistance appeared most frequently (33%) among these strains.

Table 1 Frequency of isolation of metal resistant and drug resistant isolates of *Proteus mirabilis*[1].

	Number of strains isolated in (%)		
	1975-1977	1979-1982	Total
Metal[2]			
Hg	70 (58.3)	50 (16.8)	120 (28.8)
Cd	115 (95.8)	284 (95.6)	399 (95.7)
As	67 (55.8)	171 (57.5)	238 (57.1)
Drug[3]			
SM	81 (67.5)	128 (43.1)	209 (50.1)
TC	72 (60.0)	102 (34.3)	174 (41.7)
CP	62 (51.7)	72 (24.2)	134 (32.1)
KM	48 (40.0)	75 (25.3)	123 (29.5)
Total	120	297	417

1. Results based on surveys of 417 strains of *Proteus mirabilis* from impatients. 2. Hg: $HgCl_2$, Cd: $CdCl_2$, As: Na_2HAsO_4. 3. SM: streptomycin, TC: tetracycline, CP: chloramphenicol, KM: kanamycin.

Out of 417 *P. mirabilis* isolates, 120 mercury-resistant strains were selected and examined for conjugal transferability of their resistance, and 107 R plasmids (89%) carrying this resistance could be demonstrated. R plasmids examined for resistance to mercury and four antibiotics most frequently isolated the following patterns: R(Hg; SM, TC, CP, KM), R(Hg; SM, TC, CPO), R(Hg; SM, KM) and R(Hg; SM, TC), in that order.

Hg^{2+} volatilization activity of P. mirabilis All strains of *P. mirabilis* containing Hg-resistant plasmids were tested and found to have volatilization activity of Hg^{2+}, as summarized in Table 4. In all cases, cells induced to grow in 10^{-5} M $HgCl_2$ exhibited a much higher rate of loss of $^{203}Hg^{2+}$ from the aqueous phase.

Table 2 Transfer of R plasmids from *P. mirabilis* (1st and 2nd cycle).

Donor	Resistance pattern	1st cycle				2nd cycle			
		Recipient	Selected for	Frequency	R-spectra	Recipient	Selected for	Frequency	R-spectra
JPM121	Hg	ML1410-Nx	Hg+Nx	10^{-2}	Hg	JE-17-Rif	Hg+Rif	10^{-3}	Hg
	SM, TC, CP, KM		SM+Nx	10^{-3}	SM, TC, CP, KM		SM+Rif	10^{-3}	SM, TC, CP, KM
JPM140	Hg	ML1410-Nx	Hg+Nx	10^{-4}	Hg	JE-17-Rif	Hg+Rif	10^{-4}	Hg
JPM197	Hg	ML1410-Nx	Hg+Nx	10^{-3}	Hg	JE-17-Rif	Hg+Rif	10^{-3}	Hg
	SM, TC, CP, KM		SM+Nx	10^{-3}	SM, TC, CP, KM		SM+Rif	10^{-4}	SM, TC, CP, KM
JPM251	Hg	ML1410-Nx	Hg+Nx	10^{-4}	Hg	JE-17-Rif	Hg+Rif	10^{-2}	Hg
	SM, TC, KM		SM+Nx	10^{-4}	SM, TC, KM		SM+Rif	10^{-2}	SM, TC, KM
JPM290	Hg	ML1410-Nx	Hg+Nx	10^{-2}	Hg	JE-17-Rif	Hg+Rif	10^{-3}	Hg
	SM		SM+Nx	10^{-2}	SM		SM+Rif	10^{-3}	SM

Table 3 Demonstration of R plasmids carrying mercury resistance from *P. mirabilis* and their resistance patterns of antibiotics.

Resistance pattern of isolates	Number of resistant strains	Resistance pattern of R plasmid	Number of strains with R plasmid
Hg; SM,TC,CP, KM	37	Hg; SM,TC,CP, KM	35
Hg; SM,TC,CP	25	Hg; SM,TC,CP	23
Hg; SM,TC,KM	7	Hg; SM,TC,KM	6
Hg; SM,TC	14	Hg; SM,TC	12
Hg; SM,CP	5	Hg; SM,CP	3
Hg; SM,KM	19	Hg; SM,KM	16
Hg; SM	5	Hg; SM	4
Hg; KM	2	Hg; KM	2
Hg	6	Hg	6
Total	120		107

Table 4 Mercury volatilization Rates in *P. mirabilis*.

Strain	Resistance pattern of R plasmids	Rate of volatilization[a] Induced	Uninduced
J53	sensitive	0.01	0.01
J53(R222)	Hg,SM,TC,CM	14.8	0.22
JPM120	sensitive	0.01	0.01
JPM121	Hg,SM,TC,CP,KM	17.8	0.22
JPM140	Hg	17.8	0.22
JPM197	Hg,SM,TC,CP,KM	19.9	0.15
JPM251	Hg,SM,TC,KM	14.8	0.15
JPM290	Hg,SM	12.7	0.22

[a] nmol/min per mg of cells.

DISCUSSION

Many bacteria have been isolated and found to be resistant to metal ions such as Hg, Cd, As, Pb, Co, Zn and Ni, and this resistance is often associated with a plasmid which also determines drug resistance [1~5,14,32]. Many studies seeking the connection between plasmids and drug resistance in bacteria have been conducted in the fields of epidemiology, genetics and biochemistry[1,6,18,20,30,32~34]. As a result of these studies, the origin, selection, spread and prevalence of drug-resistant bacteria have been determined by use of antibiotics. However, the few studies which have been conducted regarding metal resistance in bacteria have attempted to find a link between resistance to certain metals and resistance to several drugs within the contact of a hospital environment [25~27].

There is no doubt that these metals can pose a serious threat to the environment, but the nature of this bacterial resistance to metal has yet to be explained by epidemiological and genetic investigations. It is our belief that metal resistance occurs in such bacteria as a result of selectional factors beyond mere drug resistance. One of these factors may be the cause of environmental contamination by these metals.

We conducted previous studies of the metal resistance in 338 strains of *E. coli* derived from clinical lesions and found that the frequency of metal resistance in these isolates was higher than that of drug resistance[30]. Most of the strains exhibiting resistance to metal were both multiple-metal-resistant and multiple-drug-resistant. Furthermore, among 198 strains of mercury-resistant *E. coli*, 178 R plasmids with resistance to mercury could be demonstrated[30].

In the current investigation, in which we assayed both metal and antibiotic resistance in 417 strains of *P. mirabilis* isolated from clinical lesions, two peaks were clearly revealed in the distribution patterns of Hg, Cd and As resistances, enabling us to isolate Hg-, Cd- and As-resistant strains as well as drug-resistant strains of *P. mirabilis*. By contrast, the distribution pattern in the case of Pb resistance showed only a single peak.

The frequencies of resistance to metal in *P. mirabilis* were 28.8%, 95.7% and 57.1% of Hg, Cd and As, respectively, while the frequencies of resistance to SM, TC, CP and KM were 50.1, 41.7, 32.1 and 29.5, respectively, Similar results were obtained in isolates of *E. coli* and *K. pneumoniae*[28]. In considering the origin, selection, spread and prevalence of these metal-resistant and drug-resistant bacteria, it is important to note that the frequency of metal resistance was the same as or higher than that of drug

resistance[28~31]

The frequency of mercury resistance dropped from 58.3% during the period from 1975-1977 to 16.8% during 1979-1982, with no change in frequency of resistance to Cd and As. Similar results were obtained from strains of *E. coli*, *K. pneumoniae* and *S. aureus*[19]. At Jikei University Hospital, mercurial consumption from 1972 to 1977 averaged 2.2 kg of $HgCl_2$ per year, but after 1977, the use of mercurials was discontinued. It is proposed that selection for mercury-resistance occurred in the hospital setting when use of mercurials was widespread and that the decrease in frequency can be attributed to termination of the use of these compounds in the hospital.

We carried out mating experiments with a total of 120 strains of Hg-resistant *P. mirabilis* to examine the presence of conjugally transferable R plasmids, and among these, 107 R plasmids with mercury resistance could be demonstrated. Similar results were obtained in *E. coli* and *K. pneumoniae*[28,30,31]. It is noteworthy that transmissible R plasmids carrying Hg resistance were demonstrated in a large portion (91%) of the Hg-resistant isolates.

Next, isolates containing these mercury-resistant plasmids were assayed for the volatilization of Hg^{2+} by using radioactive $^{203}Hg^{2+}$. All of these isolates of *P. mirabilis* have inducible volatilization activity.

The presence of R plasmids in *P. mirabilis* strains is the main factor in their resistance to certain drugs and mercury. A mechanism of the plasmids' mercury resistance induces the volatilization of mercury from the growing culture. In considering the origin of these resistant plasmids, it is also important to that most of the mercury-resistant bacteria carried R plasmids with Hg resistance as well as drug resistance, and that the same mechanisms of mercury resistance occurred in *E. coli*, *K. pneumoniae*, *P. aeruginosa* and *S. aureus*.

SUMMARY

417 strains of *Proteus mirabilis* were isolated and studied for their resistance to three metals and four drugs. Distribution patterns of susceptibility clearly revealed two peaks of resistance to Hg, Cd, and As, but only one peak in the case of Pb. The frequencies of resistance to Hg, Cd, As, SM, TC, CP, and KM were 28.8, 95.7, 57.1, 50.1, 41.7, 32.1, and 29.5%, respectively. And the frequency of Hg resistance in *P. mirabilis* isolates was 58.3% during 1975 to 1977 and decreased to 16.8% in 1979 to 1982.

Among these isolates, 120 Hg-resistant strains were tested for their ability to transfer this resistance to mercury and drug-sensitive recipients of *E. coli* K12-ML1410-Nx and *E. coli* JE17-Rif. 107 of these, or 89%, demonstrated Hg-resistant transferable R plasmids.

Further, using radioactive $^{203}Hg^{2+}$, we tested the ability of strains containing these plasmids to volatilize mercury. It was found that all of these strains have inducible ability to volatilize to inorganic mercury.

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臨床分離のプロテウス菌における重金属耐性について

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薬剤耐性を支配しているプラスミドには，水銀，カドミウム，砒素，鉛といった重金属に対する耐性を同時に支配しているものが多数存在することが知られている。これらの重金属耐性菌についてはすでに多くの研究が疫学的，遺伝学的，生化学的な立場からなされている。筆者らも大腸菌，緑膿菌，黄色ブドウ球菌などの菌について，これらの生体に有害な重金属に対する耐性菌の疫学的遺伝学的検討を加え，重金属耐性菌の出現頻度が薬剤耐性のそれと同程度もしくはより高いということ，さらに水銀耐性の大部分がプラスミドによって支配されていることを明らかにした。今回は，1975年から1982年にかけて分離されたプロテウス菌を用いて，疫学的遺伝学的さらには生化学的検討を加えて，つぎの結果を得た。

1）プロテウス菌でも他の多くのバクテリアと同様，水銀，カドミウム，砒素に対する耐性値分布はきれいな2峰性のパターンを示し耐性菌と感受性菌に分けることができた。

2）プロテウス菌でもやはり重金属耐性菌の出現頻度は薬剤耐性菌のそれと同程度もしくはより高い数字を示した。

3）水銀耐性菌についてみると，120株の水銀耐性菌の89%のものがRプラスミドの上に水銀耐性の遺伝子を持っていた。すなわちほとんどの水銀耐性は大腸菌と同じようにRプラスミドによって支配されていた。

4）これらのRプラスミド支配による水銀耐性菌の耐性機構を $^{203}Hg^{2+}$ を用いて調べた結果，すべての菌で培地中からの水銀の気化が認められた。

5）1977年を境に昇汞の使用を中止したことから，それ以前と以後の水銀耐性菌の頻度を比較すると58%から17%へとほぼ3分の1に減少していた。このことから臨床由来株では病院内での水銀剤の使用が1つの選択因子になっているものと思われる。

Key wards: *Proteus mirabilis*, R plasmid, Heavy metal, Hg-resistant, Cd-resistant
プロテウス菌，Rプラスミド，重金属，水銀耐性菌，カドミウム耐性菌

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