

Characteristics of Mercury-Resistant Bacteria Isolated from River Water

Chung, Hyen-Mi, Sang-Jong Kim, and Yung-Hee Kho*

Department of Microbiology, College of Natural Sciences, Seoul National University,
Seoul 151-742, * Genetic Engineering Research Center, KIST

하천에서 분리한 수은 내성세균의 특성

정현미·김상종·고영희*

서울대학교 자연과학대학 미생물학과

* 한국과학기술원 유전공학센터

ABSTRACT: In samples taken from mouth of the Nakdong River, mercury-resistant bacteria grown on the media supplemented with over 20 ppm of mercuric chloride were below 0.3% of all aerobic heterotrophs. Among them, seven strains grown over 100 ppm of mercuric chloride were isolated and all were identified as *Pseudomonas*. The toxic effect of mercury on the growth of the most resistant strain N14 was influenced by the organic compounds and concentration. The growth and physiological activity to N14 strain were affected by toxic mercury in the early stage: The viable count and glucose turnover rate of N14 strain dropped to the lowest level as soon as the bacteria came into contact with mercury. During the extended lag period, however, bacteria accommodated to the stress and the viable count and glucose turnover rate increased. After the lag period, bacteria began to proliferate and their growth reached similar level to that of control. In crude extracts of N14 strain grown in nutrient broth containing $10 \mu\text{M HgCl}_2$, a mercuric ion dependent oxidation of NADPH was demonstrated. Therefore the mechanism of mercury-resistance of the N14 strain involved the elimination of the mercury from growth media. In the N14 strain which a wide range of resistance to antibiotics was observed in, four multiple plasmids were detected. As a result, the supposition that N14 strain has a plasmid-encoded enzyme system may be quite within the realms of possibility.

KEY WORDS □ Mercury-resistant bacteria, river water, mercuric ion reductase

The presence of heavy metals in the environment has received a great deal of attention due to their highly toxic nature and translocation through the food chain.

The problem of mercury pollution came into focus after the discovery of Minamata disease. Thereafter, ecological, biochemical and genetical analysis about the effect of mercury has been carried out.

Mercury compounds are highly toxic because of their solubility in liquid and their bonding to sulfhydryl group of proteins in membrane and enzymes.

In spite of these toxic effects, a number of mercury-resistant microorganisms were reported. A

positive correlation between the distribution of mercury compounds and that of resistant microorganisms in metal-contaminated sediment has been reported (Timony *et al.*, 1978). In the mercury-polluted sewage, soil, fresh water and seawater as well as in animal's normal flora, mercury-resistant bacteria were isolated (Austin *et al.*, 1977; Dos Reis *et al.*, 1978; Nelson and Colwell, 1975; Stickler and Thomas, 1980). These phenomena of microbial resistance is of some fundamental importance and is particularly relevant to microbial ecology, especially in the reclamation of metal-contaminated natural habitats (Gadd and Griffiths, 1978).

The present study attempts to understand the ef-

fect of mercury on the activity of mercury-resistant bacteria which were isolated from the Nakdong River, and to examine the mechanism of mercury-resistance.

MATERIALS AND METHODS

Isolation and identification of mercury-resistant bacteria

Surface water samples were taken from mouth of the Nakdong River receiving a large influx of heavy metal pollution. Dilluted samples were spread onto plates of modified ZoBell (MZ) media amended with 20-50 ppm of mercury (HgCl_2 ; MW 271.51) and incubated at 25 °C for 14 days. Three serial streakings of single colonies on the media containing mercury were done to ensure the purity and to confirm the mercury-resistance of the strains.

The isolated strains were identified to genus level according to the scheme of Shewan *et al.* (1960) and Bergey's Manual of Systematic Bacteriology (Krieg and Holt, 1984).

Culture condition

The isolated strain N14 was grown in 0.1% glucose media (GM; glucose 1g, NH_4Cl 1g, KH_2PO_4 1g, NaCl 2g, DW 1l, final pH7) on a shaker at 24 °C. The growth was assessed by the A_{410} and by viable count on MZ media.

Total bacterial number

After bacteria were stained with acridin orange, the total bacterial number was measured by epifluorescence microscopy (magnification X1875) (Zimmermann and Meyer-Reil, 1974). For high accuracy, bacteria in 30-40 fields (grid areas) were counted.

Glucose turnover rate

The method used was based on the technique of Gocke (1977). D-[^3H]-glucose (specific activity 18Ci/mM, ICN) was used and the final concentration was 0.2 ug glucose/l (0.2Ci/10 ml). The radioactivity was counted in a scintillation counter (Packard Instrument, Model 3385). The turnover rate was estimated by following equation (Williams and Askew, 1968).

$$\text{Tr} = d/Dt$$

Tr; turnover rate
d ; uptake dpm
D ; standard dpm
t ; incubation time

Mercuric reductase assay

With crude cell extracts from strain N14, the HgCl_2 -dependent oxidation of NADPH was measured by following the decrease in A_{340} (K.

Izaki, 1980; K. Izaki *et al.*, 1974).

Resistance to antibiotics

Mercury-resistant isolates were tested for resistance to ampicillin (50 ppm), chloramphenicol (10 ppm), kanamycin (50 ppm), streptomycin (25 ppm) and tetracyclin (15 ppm).

Plasmid detection and curing

The procedure used to screen for plasmid was the method of Birnboim and Doly (1979). Culture (5 ml) were grown overnight in LB medium: then 1.5 ml of bacterial suspension was harvested, lysed with solution I (50 mM glucose, 10 mM EDTA, 25 mM Tris. HCl pH 8.0) and solution II (0.2N NaOH, 1% SDS), and subjected to agarose gel electrophoresis for detection of plasmid DNA.

Strain N14 was cured of their plasmid with mitomycin C. The strain which could not grow in media supplemented with mercury was selected as cured strain.

RESULTS AND DISCUSSION

In samples taken from mouth of the Nakdong River, mercury-resistant bacteria grown on the media supplemented with over 20 ppm of mercury chloride were below 0.3% of aerobic heterotrophs. Among them, seven strains grown over 100 ppm of mercuric chloride were selected.

All seven strains were rods, motile, gram negative and oxidase positive; no pigment produced; All catabolized the carbohydrate by oxidation (Table 1). These characteristics were consistent with those of genus *Pseudomonas*. Therefore it showed that the resistance occurred among a restricted range of organisms. This result was similar to that of the Nelson and Colwell (1975) which had reported that genus *Pseudomonas* composed 66% of mercury-resistant bacteria. Among the seven isolated strains, N14 was selected as the most resistant bacteria for further study.

Because environmental factors such as concentration of organic compounds and pH influence the toxicity of mercury, pretests on media were carried out. Although N14 strain was grown rapidly in MZ media, the toxic effect was almost masked in MZ media compared with in GM media (Table 2). The growth rate in 1.0% GM media and 0.1% GM media were roughly similar, but toxic effect on bacteria in 0.1% GM media appeared more acute. Accordingly, 0.1% GM media was selected to examine toxic effect of mercury

The growth rates of N14 strain in the presence of

Table 1. Morphological and physiological characteristics of the mercury-resistant isolates

A. morphological characteristics	
1. gram stain	; negative
2. shape	; rod
3. motility	; motile
4. colony color	; white
5. growth type in broth	; dispersent
B. physiological characteristics	
1. O/F test	; oxidative
2. oxidase	; produced
3. catalase	; produced
4. nitrate reduction	; negative
5. methyl red	; negative
6. gas production	; not produced
7. gelatin liquefaction	; negative
8. starch hydrolysis	; hydrolysed
9. inositol	; negative
10. O/129	; positive

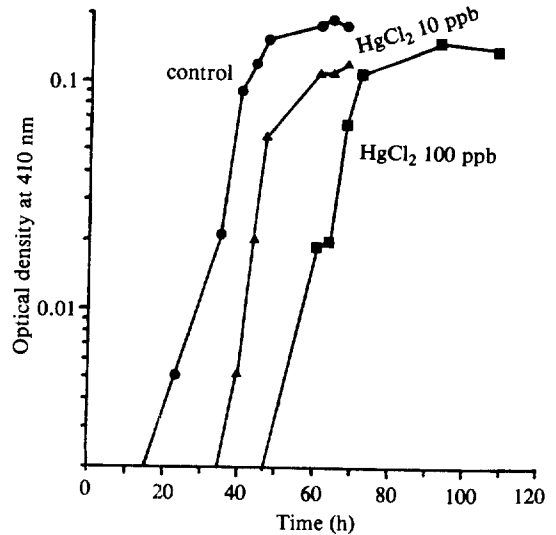
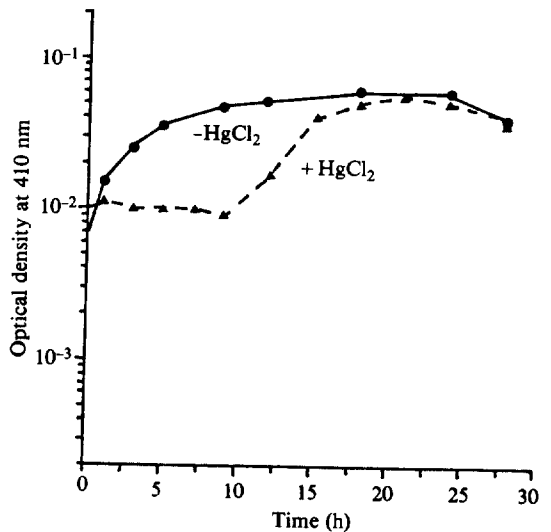
Table 2. Effect of organic composition and concentration of media on the bacterial growth and toxicity of mercury

Media	Maximum OD		Lag time (h)	
	control added*	mercury	control added*	mercury
1/10 MZ	0.300	0.032	0	0
1/100 MZ	0.045	0.045	0	5.5
1% GM	0.068	0.060	0	12
0.1% GM	0.070	0.065	0	21

(MZ; modified ZoBell media, GM; glucose minimal media *; 100 ppm of mercuric chloride added).

different concentrations of mercury were also measured (Fig. 1). Bacteria were grown at concentrations of 1 ppb, 10 ppb and 100 ppb after extended lag periods. As the concentration of mercury increased, the lag periods lengthened. At 1000 ppb, bacteria did not grow even during 100 hours incubation. Therefore the minimum inhibitory concentration of mercury was 1 ppm. On the 0.1% GM plate media, the minimum inhibitory concentration was 10 ppm.

In order to investigate the effect of mercury on bacterial activity, the following experiment was set. In 1/ of 0.1% M media supplemented with 200 ppb

**Fig. 1.** Growth of isolate N14 in the presence of mercuric chloride.**Fig. 2.** Effect of mercuric ion on turbidity of isolate N14.

of mercuric chlorid, N14 strain was inoculated (inoculum size 1.8×10^6 cells/ml) and incubated at 24°C for 28 hours. At 2-3 hours interval, aliquotes were taken and the turbidity, viable count, total bacterial number and glucose turnover rate were measured. In the initial stage, the strong toxic effect of mercury on growth and activity of bacteria was observed. That is, turbidity and total bacterial number appeared long lag time for about 9 hours (Fig. 2 and 3), viable count decreased by 99.9% of inoculum size (Fig. 4), glucose turnover rate decreased by 93.2% of inoculum size (Fig. 5). But before long,

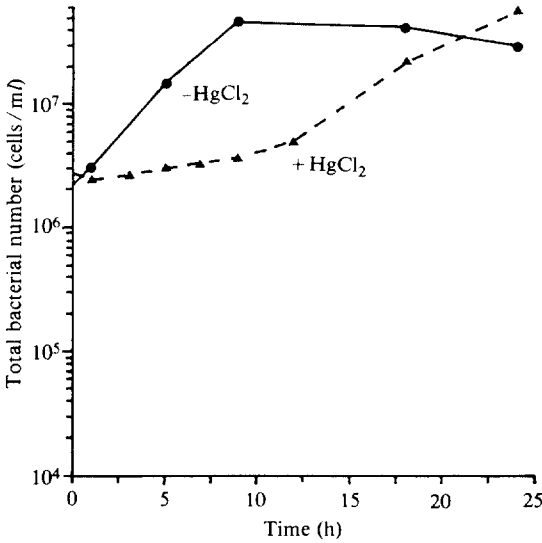


Fig. 3. Effect of mercuric ion on total bacterial number of isolate N14.

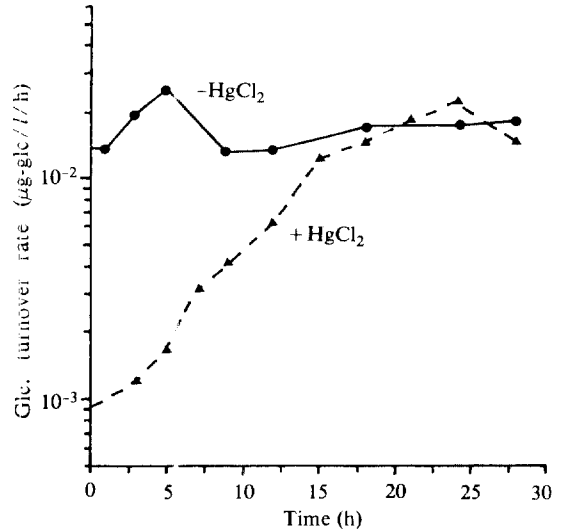


Fig. 5. Effect of Hg²⁺ on ³H-glucose turnover rate of isolate N14.

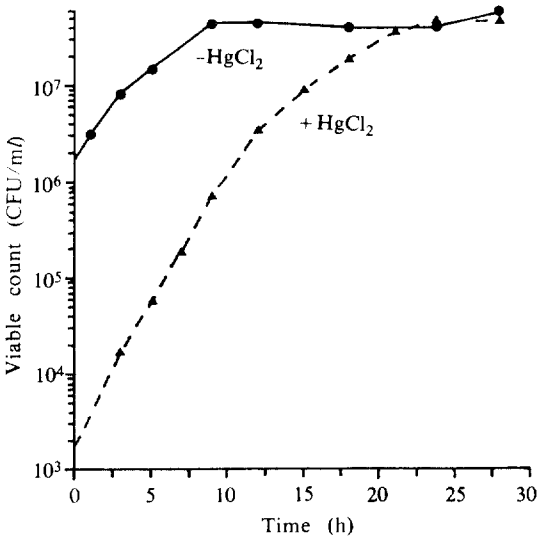


Fig. 4. Effect of mercuric ion on viable count of isolate N14.

exponential growth was observed in viable count. Glucose turnover rate also increased rapidly. After 10 hours and 15 hours, viable count and glucose turnover rate reached respectively similar level to those of control before growth. After 24 hours, the increasing rate of viable count and glucose turnover rate reduced and then they reached stationary phase. Turbidity reached stationary phase after 21 hours. In comparison with the growth of control, the phenomena of rapid decreasing of bacterial growth in the

early stage appeared as special features.

The result that bacteria had no sooner contacted with mercury than viable count decreased to 0.1% of inoculum size means that mercury was highly toxic. But the decrease of viable count does not seem to mean killing. Mitra *et al.* (1975) suggested the hypothesis that cadmium-resistant bacteria which damaged DNA by toxic Cd⁺ repaired their damage after some accommodation period. It seems likely that the decrease of viable count is loss of activity since i) growth rate of damaged bacteria ($\mu = 0.77$) is higher than that of control ($\mu = 0.51$); ii) during the lag period, cell lysis did not take place; iii) contradictorily, glucose turnover rate of one bacterium became 20 to 87 times as much as that of control, if only viable count supposed living cells. Therefore, although bacteria were inactivated and lost their ability to produce colonies when they contact with mercury, they seemed to adapt and activate in a short period time and then proliferate.

The glucose turnover rate per living cell which was counted not with viable count but with total bacterial count dropped in the early stage compared with that of control (Fig. 6). However, then it increased and reached maximum value after 9 hours. After 12 hours, it decreased and showed similar value to that of control.

Consequently, N14 strain, the mercury resistant bacteria, were affected severely by toxic mercury. And their activity and growth rate decreased in the early stage. But they recovered their activity soon and

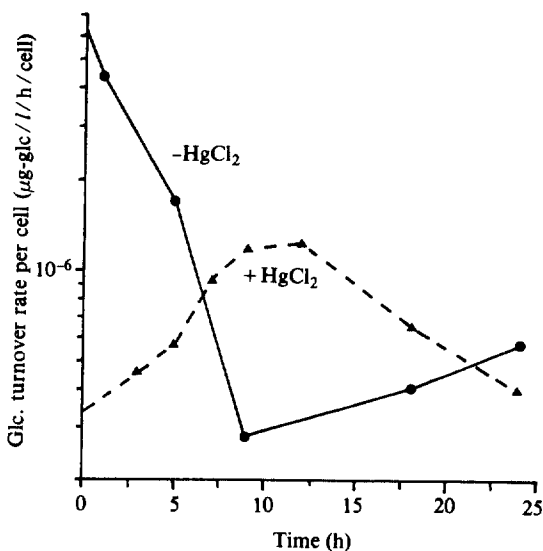


Fig. 6. Effect of mercuric ion on glucose turnover rate per bacterium.

showed similar activity and growth rate to that of control.

The mechanism of resistance to mercuric ion involves the elimination of the metals from the growth media. In all cases studied to date, mercury has been shown to be converted to volatile form which is eliminated from the growth media (Robinson and Tuovine, 1984). The volatilization of mercury is the action of the inducible mercuric reductase enzyme. The bacterial volatilization of mercury can be measured by labeled Hg, by atomic absorption spectrometer quantitatively, and by enzyme activity of mercuric reductase. In case of isolated N14 strain, enzyme activity of mercuric reductase measured by HgCl₂-dependent oxidation of NADPH (Fig. 7). NADPH in reaction mixture added mercuric chloride oxidized rapidly compared with that in the reaction mixture without mercuric chloride. Thus it was found that N14 strain had inducible mercuric reductase and it became certain that N14 strain had genes which controlled mercuric reductase.

The relationship between resistance to mercury and antibiotics in the hospital environment has been explored in numerous studies (Nakahara *et al.*, 1977). When N14 strain were tested the antibiotic resistance, it had resistance to all five antibiotics. Especially, in 250 ppm of ampicillin and kanamycin, and in 100 ppm of chloramphenicol and streptomycin N14 strain formed colonies. Therefore the resistance to these antibiotics appeared stable, and broad-spectrum resistance to antibiotics was shown in N14

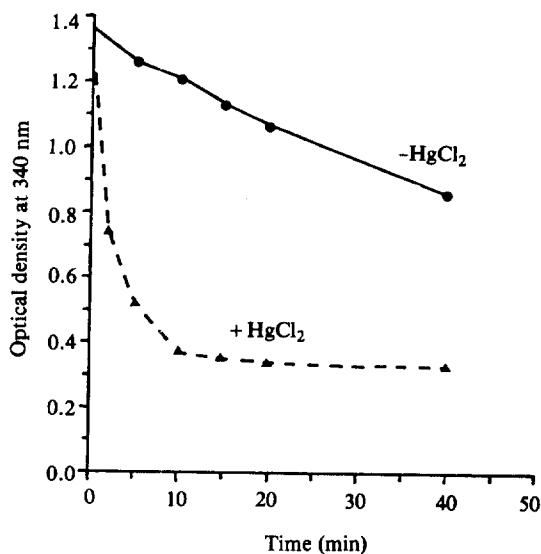


Fig. 7. NADPH oxidation dependent on Hg²⁺ by crude extract of the isolate N14 grown in the presence of HgCl₂.

strain.

In many cases, plasmids has been reported to carry mercury-resistant genetic determination, especially related to R factor (Summer and Lewis, 1973; Chackrabarty, 1976). In the case of strain N14, 4 plasmids whose size were 4.0Kb to 1.0Kb were detected. Because N14 strain had multiple plasmids, the possibility of location of mercury resistance genes in plasmids was high. To find out if mercury resistance of N14 strain was encoded by plasmid, plasmids were cured with mitomycin C known as effective curing agent of genus *Pseudomonas*. But cured strain could not be obtained. It seems to make curing difficult that the plasmids were small size and multiple. If each plasmids had been isolated and transformed to mercury sensitive strain respectively, it would have been found which plasmid had mercury resistance genes.

Eventhough the heavy metal resistant bacteria appeared in any place in which bacteria existed, they appeared in a higher frequency of existance in the place which was polluted with heavy metal (Walker and Colwell, 1974; Austin *et al.*, 1977; Kim, 1985).

Mercury-resistant bacteria N14 inactivated when they contacted with mercury. But they adapted soon and recovered their activity. They had a mechanism of reducing and volatilizing mercuric ion from surrounding environment. This mechanism can increase the possibility of bacterial survival in the presence of mercury.

Mercury-resistance of bacteria is obtained by genetic adaption. Mercury resistance genes, as other resistance genes carried by plasmids, often occurred on transposones (Bennett *et al.*, 1978). Therefore, further studies of mercury-resistant gene regulation and gene transfer mechanisms will be able to help the understanding of evolution and distribution of mer-

cury-resistant bacteria in polluted areas.

Consequently, in the point of providing the adaptation ability to bacterial community and reclamation of mercury-contaminated natural habitat, mercury-resistant bacteria are ecologically important.

적 요

남동강에서의 수은 내성세균의 분포를 조사한 결과 HgCl₂가 20ppm 이상 함유된 배지에서 성장이 가능한 수은 내성세균이 전체 호기성 중추영양세균수의 0.3% 이하로 존재하였다. 수은 내성세균 중에서 HgCl₂가 100ppm 이상 함유된 배지에서 성장이 가능한 7개 균주를 순수분리·동정된 결과 모두 *Pseudomonas* 속으로 밝혀졌다. 분리된 수은 내성세균 N14의 성장에 미치는 수은의 독성효과는 유기물의 종류와 농도에 따라 크게 영향을 받았다. 분리균주 N14의 성장과 생리적 활성도는 성장 초기단계에 수은 농도의 영향을 예민하게 받아 lag period가 길어졌다. Lag period가 끝난 후에는 세균이 증식을 시작하여 24시간의 성장은 control과 비슷한 수준에 도달하였다. 10M의 HgCl₂가 포함된 액체 배지에서 배양한 균주 N14의 추출물에서는 Hg ion에 의존하는 NADPH의 산화가 확인되었다. 넓은 범위의 환경제에 대해 내성을 보이는 분리균주 N14에서는 4개의 multiple plasmids가 발견되어 plasmid에 의해 coding되는 효소계의 존재 가능성을 시사한다.

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