Mercury Resistance and R Plasmids in *Escherichia coli* Isolated from Clinical Lesions in Japan

HIDEOMI NAKAHARA,¹ TOMOAKI ISHIKAWA,² YASUNAGA SARAI,³ ISAMU KONDO, HIROYUKI KOZUKUE,* AND SUSUMU MITSUHASHI

Departments of Hygiene* and Microbiology, The Jikei University School of Medicine, Tokyo 105, Japan; and Department of Microbiology, School of Medicine, Gunma University, Maebashi 371, Japan.

Received for publication 26 October 1976

The mercury and antibiotic resistance of 338 strains of *Escherichia coli* isolated from hospital patients was determined. Resistance to mercury was found in 58.6% of the isolates. The frequencies of resistance to streptomycin (Sm), tetracycline (Tc), chloramphenicol (Cm), kanamycin (Kan), cephaloridine (Cer), and gentamicin (Gm) were 66.3, 60.3, 56.5, 42.9, 32.1, and 1.5%, respectively. Among the above, 198 mercury- and antibiotic-resistant isolates were selected and tested for their ability to transfer the resistance to susceptible strains of *E. coli* K-12 and *Klebsiella pneumoniae* JK5. R plasmids carrying mercury resistance were demonstrated in 89.9% of the mercury-resistant strains of *E. coli*. Furthermore, R(Hg,Sm,Tc,Cm) plasmids were demonstrated most frequently, followed by R(Hg,Sm,Tc,Cm,Kan), R(Hg,Cm,Kan), and R(Hg,Sm,-Tc,Cm,Kan,Cer) plasmids.

Resistance of bacteria to mercury has been described by several authors from the viewpoints of morphology (23), genetics, and biochemistry. In many bacteria, resistance to mercury is associated with a plasmid (7, 8, 13, 14, 16, 17, 19). Generally, microorganisms have been shown to detoxify mercurial compounds metabolically by forming either volatile mercury derivatives (1, 12, 16, 19, 22) or mercury mercaptides (5, 15, 18, 25). There are also many bacteria that are resistant to other metallic ions such as cadmium, arsenate, lead, zinc, cobalt, and nickel (8-10, 13, 17).

It is of interest that resistance to these metals is mediated by the same plasmid that determines resistance to antibiotics. Also, most of these metals have recently been listed as established or possible causes of environmental pollution. For example, methyl mercury is known to cause Minamata disease (6, 20), and cadmium is the causative agent of Itai-Itai disease in Japan.

The role of R plasmids in drug resistance has been widely studied (2-4, 11, 21, 24), and extra-chromosomal determinants are a main cause of the increase in numbers of drug-resistant bacteria. However, the factors selecting for these metal-resistant bacteria have not yet been

identified. We believe that metal-resistant microorganisms do not arise by chance, but that there must be selection factors beyond mere drug resistance. One of these selection factors may be environmental contamination by these heavy metals.

We previously investigated the distribution of the resistance to several metallic ions such as Hg, Cd, As, and Pb among 415 strains of *Staphylococcus aureus* (*S. aureus*) isolated from hospital patients (9). Recently, we also demonstrated 198 R plasmids with Hg resistance in *Escherichia coli* isolated from clinical specimens. This paper deals with the genetic properties of these R plasmids carrying Hg resistance.

MATERIALS AND METHODS

Bacterial strains. A total of 338 strains of *E. coli* were isolated from the Clinical Laboratory of The Jikei University Hospital and tested for their resistance to mercury and six antibiotics. Mating experiments were carried out with a total of 198 Hg- and antibiotic-resistant strains as donors to examine the presence of conjugally transferable R plasmids. *E. coli* K-12 ML1410 and *Klebsiella pneumoniae* JK5 were used as recipient bacteria. *E. coli* K-12 ML1410 was kindly supplied by Matsuhisa Inoue, Department of Microbiology, School of Medicine, Gunma University. It is a nalidixic acid- and rifampin (Rif)-resistant mutant of *E. coli* K-12. *K. pneumoniae* JK5 was isolated at our laboratory.

Drug resistance tests. Minimal inhibitory concentrations of the six antibiotics tested against all *E. coli* strains were determined by the agar dilution method using nutrient agar. Antibiotics used were streptomycin (Sm), tetracycline (Tc), chlorampheni-

¹ Present address: Department of Biology, Washington University, St. Louis, MO 63130.

² Present address: Department of Internal Medicine, The Third Hospital, The Jikei University School of Medicine, Izumi, Komae, Tokyo 182, Japan.

³ Present address: Department of Pediatrics, National Medical Center Hospital of Japan, Tokyo 162, Japan.

col (Cm), kanamycin (Kan), cephaloridine (Cer), and gentamicin (Gm). Those cultures not inhibited by 25 μ g of Sm, Tc, Cm, or Kan per ml or by 12.5 μ g of Cer or Gm per ml were regarded as resistant to each of the antibiotics.

Mercury resistance tests. As the mercury derivative, we used HgCl_2 . HgCl_2 was dissolved in distilled water, sterilized by membrane filtration (Millipore Corp.; pore size, 0.45 μ m), and added to agar medium in the designated concentrations.

Conjugation experiments. Donor strains of *E. coli* were cultivated for transfer in nutrient broth (Difco) with shaking for 5 h. One milliliter of a culture containing about 10^6 bacteria was mixed with 1 ml of a similarly prepared recipient culture. The mixture was statically incubated for 4 to 8 h, and then about 0.01 ml was plated on Simmons citrate agar (Eiken) or nutrient agar (Difco) containing 100 μ g of Rif per ml plus one of the following substances: 25 μ g of Sm, 25 μ g of Tc, 25 μ g of Cm, or 10 μ g of HgCl_2 per ml. The plates were incubated overnight at 37°C and then compared with control plates with individual antibiotics and the parental strains, and also with Rif plates plus single-antibiotic or Hg additions.

RESULTS

Distribution of Hg- and antibiotic-resistant strains of *E. coli*. Figure 1 shows the distribution curve of Hg resistance obtained by the agar dilution method from 338 clinical isolates of *E. coli*. The bimodal distribution curve of susceptibility to Hg showed a clear-cut distinction between susceptible and resistant populations, and it can be seen that the resistance was differentiated by concentrations of HgCl_2 above 10 μ g/ml.

The frequency of resistance to Hg was 58.6% among the 338 isolates. On the other hand, the frequencies of resistance to Sm, Tc, Cm, Kan, Cer, and Gm were 66.3, 60.3, 56.5, 42.9, 32.1, and 1.5%, respectively.

Transfer of plasmids carrying Hg resistance. In the first cycle of transfer, the first five strains of *E. coli*, i.e., JE504, JE456, JE333, JE335, and JE224, transferred the whole complex pattern of resistance at a frequency of about 10^{-3} to *K. pneumoniae* JK5 (Table 1). However, exconjugants from crosses of the remaining strain, JE117, were resistant to Hg and all of the antibiotic markers except Kan, the latter apparently segregating out.

These exconjugants were also tested for transfer of their resistance to *E. coli* K-12 ML1410-Rif as the recipient. The resulting exconjugants were selected on nutrient agar with 100 μ g of Rif per ml plus a single selecting drug or Hg in the concentrations given above. After 3 h or more of mating, a high frequency of transfer of virtually the total resistance pattern to the *E. coli* K-12 recipient was observed.

FIG. 1. Distribution pattern of resistance to Hg in 338 strains of *E. coli*. The Hg resistance revealed a clear-cut bimodal distribution. From this pattern, those cultures not inhibited by 10 μ g of Hg per ml were regarded as resistant to Hg.

Some data concerning this second cycle of transfer are given in Table 1. This pattern of resistance was then well transferable in further cycles from and to the recipient strains described.

Resistance patterns of Hg-resistant *E. coli* strains. Resistance patterns of *E. coli* strains possessing Hg resistance are shown in Table 2. Triple-drug resistance appeared most frequently (37.9%) among the strains carrying Hg resistance, followed by quadruple (26.8%)-, double (24.2%)-, and quintuple (7.6%)-drug resistance. In contrast, among these strains, those carrying single- and sextuple-drug resistance were 2 and 1.5%, respectively. Strains carrying only Hg resistance have not been isolated as yet from *E. coli*.

Genetic properties of the R plasmids carrying Hg resistance. Among the 338 isolates of *E. coli*, 198 strains resistant to Hg were selected, and conjugal transferability of their resistance was examined. Among these 198 Hg-resistant strains, 178 R plasmids carrying Hg resistance (89.9%) could be demonstrated (Table 2). With reference to resistance to Hg and/or six antibiotics, R plasmids with (Hg; Sm, Tc, Cm) resistance were isolated most frequently, followed by R(Hg; Tc, Cm, Kan), R(Hg; Sm, Kan), and R(Hg; Sm, Tc, Cm, Kan, Cer), in that order.

The frequencies of strains with resistance to Sm, Tc, Cm, Kan, Cer, and Gm mediated by R plasmids carrying Hg resistance were 88.8, 86.0, 82.6, 51.1, 10.7, and 1.7%, respectively.

DISCUSSION

It is well known that R plasmids in *E. coli* carry genes determining resistance to several metallic ions such as Hg, Co, and Ni (17). Also, penicillinase plasmids mediating resistance to Hg, Cd, As, Pb, and Zn have been observed in *S. aureus* (13, 14). We have reported that the resistances to Hg and Cd mediated by a penicillinase plasmid were found to be controlled by quite different mechanisms (8).

Numerous studies of drug-resistant bacteria have clarified the close relationship between the appearance of drug-resistant bacteria and usage of the drug. However, the nature of the selection factor for these metal-resistant bacteria has not yet been fully explained from the epidemiological and genetic viewpoints. We have assumed that these metallic ions themselves could be the selection factor because of environmental contamination by these metals.

TABLE 1. Transfer of R plasmids from *E. coli* (first and second cycles)

Donor	Resistance pattern	1st cycle			2nd cycle			Resistance spectrum
		Recipient	Selected for:	Frequency	Recipient	Selected for:	Frequency	
JE504	Hg Sm, Tc, Cm Kan, Cer, Gm	JK5	Hg + Cit ^a Sm + Cit	10 ⁻³ 10 ⁻³	ML1410-Rif ML1410-Rif	Hg + Rif ^b Sm + Rif	10 ⁻⁴ 10 ⁻³	Hg; Sm, Tc, Cm, Kan, Cer, Gm Hg; Sm, Tc, Cm, Kan, Cer, Gm
JE456	Hg Sm, Tc, Cm Kan, Cer	JK5	Hg + Cit Sm + Cit	10 ⁻³ 10 ⁻³	ML1410-Rif ML1410-Rif	Hg + Rif Sm + Rif	10 ⁻³ 10 ⁻³	Hg; Sm, Tc, Cm, Kan, Cer Hg; Sm, Tc, Cm, Kan, Cer
JE333	Hg Sm, Tc, Cm	JK5	Hg + Cit Sm + Cit	10 ⁻³ 10 ⁻³	ML1410-Rif ML1410-Rif	Hg + Rif Sm + Rif	10 ⁻⁴ 10 ⁻³	Hg; Sm, Tc, Cm Hg; Sm, Tc, Cm
JE335	Hg Tc, Cm	JK5	Hg + Cit Tc + Cit	10 ⁻³ 10 ⁻⁴	ML1410-Rif ML1410-Rif	Hg + Rif Tc + Rif	10 ⁻² 10 ⁻⁴	Hg; Tc, Cm Hg; Tc, Cm
JE224	Hg Cm, Kan	JK5	Hg + Cit Cm + Cit	10 ⁻³ 10 ⁻⁴	ML1410-Rif ML1410-Rif	Hg + Rif Cm + Rif	10 ⁻³ 10 ⁻⁴	Hg; Cm, Kan Hg; Cm, Kan
JE117	Hg Sm, Tc, Cm Kan, Cer	JK5	Hg + Cit Sm + Cit	10 ⁻³ 10 ⁻⁴	ML1410-Rif ML1410-Rif	Hg + Rif Sm + Rif	10 ⁻³ 10 ⁻³	Hg; Sm, Tc, Cm, Cer Hg; Sm, Tc, Cm, Cer

^a Cit, Simmons citrate agar containing 10 µg of HgCl₂ per ml.

^b Nutrient agar containing 100 µg of Rif plus 10 µg of HgCl₂ per ml.

TABLE 2. Demonstration of R plasmids carrying Hg resistance from *E. coli* and their resistance patterns of antibiotics

Resistance pattern	No. of resistant strains	Resistance pattern of R plasmid transferred	No. of strains with R plasmid
Hg; Sm, Tc, Cm, Kan, Cer, Gm	3	Hg; Sm, Tc, Kan, Cer, Gm	3
Hg; Sm, Tc, Cm, Kan, Cer	15	Hg; Sm, Tc, Cm, Kan, Cer Hg; Sm, Tc, Cm, Cer	13 2
Hg; Sm, Tc, Cm, Kan	52	Hg; Sm, Tc, Cm, Kan	47
Hg; Sm, Tc, Kan, Cer	1	Hg; Sm, Tc, Kan, Cer	1
Hg; Sm, Tc, Cm	62	Hg; Sm, Tc, Cm	58
Hg; Sm, Tc, Kan	8	Hg; Sm, Tc, Kan	6
Hg; Tc, Cm, Kan	5	Hg; Tc, Cm, Kan	4
Hg; Sm, Tc	6	Hg; Sm, Tc	6
Hg; Sm, Cm	6	Hg; Sm, Cm	5
Hg; Sm, Kan	19	Hg; Sm, Kan	15
Hg; Cm, Kan	2	Hg; Cm, Kan	2
Hg; Tc, Cm	15	Hg; Tc, Cm	12
Hg; Sm	2	Hg; Sm	2
Hg; Tc	1	Hg; Tc	1
Hg; Cm	1	Hg; Cm	1

We have reported the distribution of resistance to Hg, Cd, As, and Pb among 415 isolates of *S. aureus*. The results were as follows. (i) The frequency of the metal-resistant strains was actually higher than that of drug-resistant strains. (ii) Most of metal-resistant strains were found to be multiple-metal resistant and also multiple-drug resistant. (iii) About 10% of the total isolates were found to be metal resistant but drug susceptible. (iv) *S. aureus* MS1396 and JS76 were found to contain a new plasmid that controls only metal resistance (9).

The distribution curve of Hg resistance clearly revealed two peaks, and as a result of this we could isolate Hg-resistant *E. coli* strains as well as antibiotic-resistant strains. From the viewpoint of the origin of Hg- and drug-resistant bacteria and their selection, spread, and prevalence, it should be noted that the frequency of Hg resistance was same as or higher than that of drug resistance. A similar result was found in *S. aureus*.

Among the strains possessing Hg resistance, those carrying triple-drug resistance were isolated most frequently, followed by quadruple-, double-, and quintuple-drug resistance. Furthermore, most of the Hg-resistant strains had joint resistance to Sm, Tc, and Cm, and it would seem that the gene for Hg resistance in these *E. coli* strains is on the same R plasmid as the Sm, Tc, and Cm resistance genes.

From the mating experiments with a total of 198 Hg-resistant strains as donors, 178 with R plasmids (89.9%) with Hg resistance could be demonstrated. In addition, among the above, 5 Hg- and antibiotic-resistant isolates were selected and tested for the elimination of Hg and antibiotic resistance. All Hg and antibiotic resistance determinants in five strains were co-eliminated at high frequency by treatment with acriflavine and growth at 43°C (about 2.4 to 15.2%).

The resistance patterns of R plasmids were quite similar to those of the resistant strains that were isolated frequently, indicating that Hg and antibiotic resistances in *E. coli* strains are mainly due to the presence of R plasmids.

ACKNOWLEDGMENTS

We are grateful to S. Silver and R. R. Colwell for their valuable and helpful discussions. We thank K. Kurosaka, K. Machida, T. Sawahata, and K. Itoh for supplying the clinical isolates.

This work was supported by a Grant-in-Aid for Scientific Research of the Japanese Ministry of Education (no. 177156).

LITERATURE CITED

- Ben-Bassat, D., G. Shelef, N. Gruner, and H. Shuval. 1972. Growth of *Chlamydomonas* in a medium containing mercury. *Nature* (London) 240:43-44.
- Curtiss, R., III. 1969. Bacterial conjugation. *Annu. Rev. Microbiol.* 23:69-136.
- Curtiss, R., III, L. J. Charamella, D. R. Stallions, and J. A. Mays. 1968. Parental functions during conjugations in *Escherichia coli* K-12. *Bacteriol. Rev.* 32:320-348.
- Datta, N., and R. W. Hedges. 1971. Compatibility groups among f^+ R factors. *Nature* (London) 234:222-223.
- Hamdy, M. K., and O. R. Noyes. 1975. Formation of methyl mercury by bacteria. *Appl. Microbiol.* 30:424-432.
- Hirayama, K., and H. Takahashi. 1970. Studies on the treatment for methyl mercury poisoning "lowing of the methyl mercury content in the poisoned animal brain". *Kumamoto Med. J.* 23:56-64.
- Iyobe, S., K. Hasuda, H. Sugai, and S. Mitsuhashi. 1975. Drug resistance and R factors in *P. aeruginosa*, p. 321-327. In S. Mitsuhashi and H. Hashimoto (ed.), *Microbial drug resistance*. University of Tokyo Press, Tokyo.
- Kondo, I., T. Ishikawa, and H. Nakahara. 1974. Mercury and cadmium resistances mediated by the penicillinase plasmid in *Staphylococcus aureus*. *J. Bacteriol.* 117:1-7.
- Kondo, I., H. Nakahara, and T. Ishikawa. 1975. The possibility of a specific plasmid mediating metal resistances, p. 145-152. In S. Mitsuhashi and H. Hashimoto (ed.), *Microbial drug resistance*. University of Tokyo Press, Tokyo.
- Mitra, R. S., R. H. Gray, B. Chin, and I. A. Bernstein. 1975. Molecular mechanisms of accommodation in *Escherichia coli* to toxic levels of Cd^{4+} . *J. Bacteriol.* 121:1180-1188.
- Mitsuhashi, S. 1971. Drug-resistance of gram-negative bacteria isolated in Japan, p. 7-16. In S. Mitsuhashi (ed.), *Transferable drug resistance factor R*. University of Tokyo Press, Tokyo.
- Nelson, J. D., Jr., W. Blair, F. E. Brinckman, R. R. Colwell, and W. P. Iverson. 1973. Biodegradation of phenylmercuric acetate by mercury-resistant bacteria. *Appl. Microbiol.* 16:321-326.
- Novick, R. P., and C. Roth. 1968. Plasmid-linked resistance to inorganic salts in *Staphylococcus aureus*. *J. Bacteriol.* 95:1335-1342.
- Richmond, M. H., and M. John. 1964. Co-transduction by a staphylococcal phage of the genes responsible for penicillinase synthesis and resistance to mercury salts. *Nature* (London) 202:1360-1361.
- Ross, I. S., and K. M. Old. 1973. Thiol compounds and resistance of *Pyrenophora avenae* to mercury. *Trans. Br. Mycol. Soc.* 60:301-310.
- Schottel, J., A. Mandal, D. Clark, S. Silver, and R. W. Hedges. 1974. Volatilisation of mercury and organomercurials determined by inducible R-factor systems in enteric bacteria. *Nature* (London) 251:335-337.
- Smith, D. H. 1967. R factors mediate resistance to mercury, nickel and cobalt. *Science* 156:1114-1116.
- Stutzenberger, F. J., and E. O. Bennett. 1965. Sensitivity of mixed populations of *Staphylococcus aureus* and *Escherichia coli* to mercurials. *Appl. Microbiol.* 13:570-574.
- Summers, A. O., and S. Silver. 1972. Mercury resistance in plasmid-bearing strains of *Escherichia coli*. *J. Bacteriol.* 112:1228-1236.
- Takizawa, Y., T. Kosaka, and R. Sugai. 1972. Studies on the cause of Niigata episode of Minamata disease outbreak. *Acta Med. Biol.* (Niigata) 19:193-206.
- Tanaka, T., A. Kobayashi, K. Ikemura, H. Hashimoto, and S. Mitsuhashi. 1974. Drug resistance and distribution of mercury. *Nature* (London) 240:43-44.

- buton of R factors among *Escherichia coli* strains. Jpn. J. Microbiol. 18:343-347.
22. Tonomura, K., K. Maeda, F. Futai, T. Nakagami, and M. Yamada. 1968. Stimulative vaporization of phenylmercuric acetate by mercury-resistant bacteria. Nature (London) 217:644-646.
23. Vaituzis, Z., J. D. Nelson, Jr., L. W. Wan, and R. R. Colwell. 1975. Effects of mercuric chloride on growth and morphology of selected strains of mercury-resistant bacteria. Appl. Microbiol. 29:275-286.
24. Watanabe, T. 1963. Infective heredity of multiple drug resistance in bacteria. Bacteriol. Rev. 27:87-115.
25. Wood, J. M., F. S. Kennedy, and C. G. Rosen. 1968. Synthesis of methylmercury compounds by extracts of a methanogenic bacterium. Nature (London) 220:173-174.