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R Factors Mediate Resistance to Mercury, Nickel, and Cobalt

Abstract. *Fifty-five clinical isolates and laboratory stocks of* Escherichia coli *and* Salmonella *were studied for resistance to each of ten metals. Eleven clinical isolates carrying R factors were resistant to mercury, and, in each case, the resistance was mediated by a previously undefined R-factor gene. The gene was phenotypically expressed within 2 to 4 minutes after entry into sensitive bacteria, but the basis for the resistance remains undefined. Fourteen strains, 12 infected with R factors, were resistant to cobalt and nickel, but these resistances were mediated by R-factor genes in only two strains; separate R-factor genes mediated the resistances to nickel and cobalt. These and other results indicate that the genetic composition of R factors is greater than that originally defined.*

The R factors are episomes of Enterobacteriaceae described originally as mediating resistance to several commonly used antibacterial drugs. Several recent findings indicate, however, that R factors may contain genes mediating other characteristics. Chemical and biological studies suggest that the DNA of a single R factor has a molecular weight of approximately 25 million daltons, an amount sufficient to code for several dozen proteins (*1*). During studies that demonstrated that the DNA of segregant R factors is reduced in quantity and altered in composition relative to the DNA of the parent episome, certain segregant R factors that had lost only four known genetic characters were found to have lost 45 percent of their DNA content (*2*); segregant R factors that had lost a significant fraction of their DNA without loss of genetic characters have also been isolated (*1*). Moreover, certain R factors have separate genes that mediate altered susceptibility to colicins, ultraviolet light, and certain experimental antibiotics and chemical inhibitors of bacteria (*3*). My data indicate that R factors may contain previously undefined genes that mediate resistance to mercury, nickel, and cobalt.

Twelve laboratory strains and 43 local clinical isolates of *Escherichia coli* and *Salmonella*, including 20 strains infected with previously defined R factors, were screened for resistance to various metals. Salts of the following metals were incorporated into enriched agar prepared with distilled water further purified by passage through a cation-exchange resin: aluminum, cadmium, chromium, cobalt, copper, lead, mercury, nickel, silver, and thallium. The ability of the strains to form colonies was tested; strains growing in three or more times the concentration of metal that inhibited most strains were defined as resistant. The techniques used for conjugation and transduction, for selection of R-factor segregants, and for the temporary alteration of cell permeability by ethylenediamine tetraacetate (EDTA) have been described (*4*).

All strains tested were equally resistant to aluminum, cadmium, chromium, copper, lead, silver, and thallium. At 1×10^{-5} mole/liter, mercury inhibited all laboratory strains and drug-sensitive clinical isolates; 11 of 20 strains carrying R factors were resistant to $1.2 \times 10^{-4}M$ mercury (Table 1). Ten of the strains were *E. coli*, and one was *Salmonella typhimurium*. Strains resistant to drugs and mercury were mated with suitable recipients, and drug resistant recombinants were selected; in each case, bacteria that acquired drug resistance mediated by the R factor also acquired resistance to mercury. Conversely, recipients selected for the acquisition of resistance to mercury also acquired resistance to drugs. When these recipients were used as R-factor donors in subsequent matings, resistance to drugs and mercury were transferred coordinately. Furthermore, resistance to mercury was transduced along with drug resistance mediated by R-factor genes but not with the several chromosomal genes tested; and bacteria selected for the spontaneous loss of drug resistance mediated by R factors also had lost resistance to mercury. These results indicated that R factors may contain a gene or genes mediating resistance to mercury.

The following findings indicated that this gene has no previously defined expression. Resistance to mercury was mediated by R factors of fi^- and fi^+ types and with varying patterns of drug resistance. Segregant clones that had lost mercury resistance but that had the pattern of drug resistance of the parent episome could be selected from cultures of *E. coli* or *S. typhimurium*.

Several factors may affect the degrees of drug resistance mediated by R factors: the medium, the episome, host strain, and, in those instances in which resistance is mediated by inactivation of a drug, the concentration of the inoculum. Resistance to tetracycline conferred by R factors is ex-

Table 1. Concentrations of mercury that inhibit certain enterobacteriaceae. Bacteria with and without R factors were tested for mercury resistance as described in the text; the minimum inhibitory concentration prevented colony formation on agar plates and growth in broth. To determine that the treatment with EDTA did alter cell permeability, I divided the treated cultures and exposed them to either mercury or actinomycin D at 10 μg/ml. Actinomycin D prevented growth of the treated cells but did not affect untreated cells.

Strain	Episome	Inoculum (No./ml)	Medium	Minimal inhibitory concentrations (mole/liter)
		No previous treatment		
E. coli B		5×10^6	Enriched	1×10^{-5}
E. coli B	E1	5×10^6	Enriched	2.5×10^{-4}
E. coli B	E1	5×10^2	Enriched	1.2×10^{-4}
E. coli B	E1	5×10^6	Minimal	3×10^{-6}
E. coli B	E7	5×10^6	Enriched	2.5×10^{-4}
S. typhimurium LT_2		5×10^6	Enriched	1×10^{-5}
S. typhimurium LT_2	E1	5×10^6	Enriched	2.5×10^{-4}
		Previous treatment with EDTA		
E. coli B		5×10^6	Enriched	1×10^{-5}
E. coli B	E1	5×10^6	Enriched	2.5×10^{-4}

pressed only after a period of exposure that varies directly with the drug concentration. The degree of resistance to mercury was affected by the medium but was relatively independent of the episome, host strain, and the concentration of the inoculum (Table 1); resistance was expressed without a lag when mercury was added to an exponentially growing culture of bacteria carrying a Hg^R-R factor. An alteration in permeability might be presumed to be the mechanism for this resistance, but certain findings suggest the possibility of other mechanisms. Expression of resistance due to an alteration in permeability might be expected to require a long lag between entry of the Hg^R gene and its phenotypic expression due to dilution of "sensitive surface receptors." However, resistance was expressed within 2 to 4 minutes after Plkc transducing phage prepared in *E. coli* carrying a Hg^R-factor was added to a culture of sensitive bacteria (Fig. 1). Although these results might only reflect that the interval between the exposure to mercury and its bacteriocidal effect exceeds that required for the phenotypic expression of resistance to mercury, $1 \times 10^{-4}M$ mercury killed > 99.99 percent of the cells within 5 minutes when added to a culture of sensitive *E. coli* in exponential growth. Furthermore, treatment with EDTA of *E. coli* carrying a Hg^R-factor did not affect the level of mercury resistance, although it did alter permeability, as evidenced by an enhanced susceptibility to actinomycin D. It seems reasonable to assume, therefore, that the expression of the Hg^R gene can occur within minutes after the introduction of the gene and that it may not be due to altered permeability.

Forty-one strains were inhibited by concentrations of Ni and Co greater than $10^{-3}M$; 14 strains, including 12 with R factors, formed colonies on agar containing either metal at 3×10^{-3} mole/liter. Only two *E. coli* strains, both infected with R factors, transferred these resistances to suitable recipients by conjugation; in each case, resistance to nickel and cobalt was transferred or lost coordinately with the pattern of drug and mercury resistance of the R factor. Attempts to demonstrate the conjugational transfer of nickel and cobalt resistance from the other ten strains with R factors have been unsuccessful, and segregants that had lost resistance to drugs and mercury without losing resistance to nickel and cobalt were isolated from several of these strains. These results indicate that a gene or genes mediating nickel or cobalt resistance may be associated with R factors in certain strains but not in others.

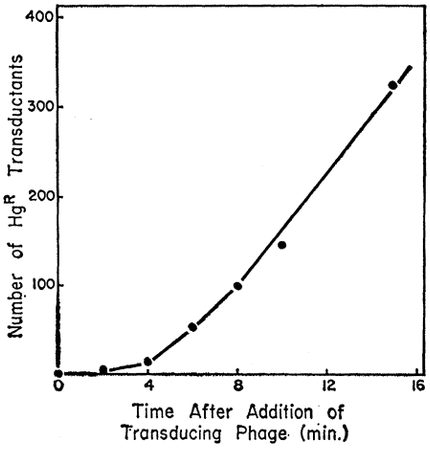

Fig. 1. The kinetics of the phenotypic expression of the R factor Hg^R gene. Plkc transducing phage prepared on *Escherichia coli* carrying a Hg^R-R factor was added to *E. coli* AB 1932 in broth at time zero; the mixture contained 4×10^9 bacteria and 5×10^8 plaque-forming virus particles per milliliter. Portions were removed at intervals and plated on enriched agar containing $1 \times 10^{-4}M$ mercury. The colonies were counted after 48 hours of incubation. *Escherichia coli* AB 1932 infected with Plkc prepared on a strain carrying an R factor that was phenotypically sensitive to mercury did not grow on the mercury agar plates.

As in the case of mercury resistance, the degree of resistance to nickel and cobalt mediated by the R factors was relatively independent of the inoculum, host strain, or episome. Both R factors mediating these resistances were fi− type and conferred identical patterns of drug and mercury resistance. The parent strains were isolated from different individuals, however, and other R factors in these series had identical genotypes without genes for resistance to nickel and cobalt. That segregant clones that had lost only resistance to nickel, or cobalt, or both could be isolated from cultures of *E. coli* or *S. typhimurium* infected with these R factors indicated that separate genes mediated the resistance to each metal.

Environmental exposure to commercial antibacterial substances has been assumed to play the major role in the selection of R-factor genes, and the introduction of certain antibiotics, for example, kanamycin and ampicillin, has been followed by the isolation of bacteria infected with R factors mediating resistance to those drugs. The present study was originally undertaken because of the high probability that bacteria are naturally exposed to many heavy metals; indeed, I anticipated that the genetic composition of R factors might reflect, in part, the pattern and extent of man's contamination of his environment with antibacterial agents. In this regard, the staphylococci are often infected with an extrachromosomal element that may contain genes mediating resistance to penicillin, and occasionally other antibiotics, and such metals as mercury, cadmium, and arsenic (*5*). These R factors of local origin are not unique, for I have found a similar gene mediating resistance to mercury on R factors infecting bacteria isolated in Chicago, Seattle, and London, and on some of the earliest known R factors, 222 and N_1 (*6*), isolated in Japan.

Other studies from my laboratory suggest, however, that the interrelationship between man's contamination of his environment and the genetic composition of R factors may not be as direct as originally presumed. Thus, R factors mediating resistance to streptomycin have been identified in bacteria isolated prior to the introduction of this antibiotic (*3*). Furthermore, the R factors in 11 of the strains used in this study have previously undefined genes that mediate resistance to two aminoglycoside antibiotics that have not yet been commercially introduced (*3*); similar genes have also been identified on the R factors 222, N_1, N_3, N_6, and N_9 isolated in Japan several years ago (*6*).

These results, and those outlined above, indicate that the genetic composition of R factors is greater than that defined originally. The origin of R-factor genes, the factors responsible for their selection, their role in the genetic potential and evolution of enteric bacteria, and the biochemical basis for their expression need definition and have important implications for biology and medicine.

Note added in proof: After this manuscript had been completed, Dr. Richard Novick informed me that he has also found that certain R factors mediate resistance to mercury (*5*).

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Anaerobic Biodegradation of DDT to DDD in Soil

Abstract. *DDT labeled with carbon-14 was added to soil, and the mixture was incubated anaerobically for 2 weeks and 4 weeks. DDT and seven possible decomposition products were separated by thin-layer chromatography, and the radioactivity of material from individual spots was determined by liquid scintillation. The DDT was dechlorinated by soil microorganisms to DDD, and only traces of other degradation products were detected. No degradation of DDT was detected in sterile soil.*

Persistence of some chlorinated insecticides in soils is well documented (*1*), and methods for accelerating decay of these materials in soils would be of monumental importance whenever an undesirable accumulation occurs. In this study, results showed that a considerable amount of DDT (*2*) was converted rather rapidly to DDD, and only 57 percent of the initial DDT was recovered in identifiable products after 4 weeks of incubation.

A number of investigators studied the conversion of DDT to DDD in animals by microorganisms. DDT was dechlorinated to DDD by *Serratia marcescens* isolated from stable flies (*3*), *Escherichia coli* and *Aerobacter aerogenes* isolated from the gastrointestinal tract of rats (*4*), and *Proteus vulgaris* from the intestinal flora of a mouse (*5*). The anaerobic condition required for the conversion process was shown by Stenersen (*3*). Wedemeyer (*6*), using sonically disrupted cell suspensions of *Aerobacter aerogenes* and selected metabolic inhibitors, showed that DDT was dechlorinated in the presence of reduced Fe (II) cytochrome oxidase. DDT was converted to DDD by yeast (*7*) and shake-cultures of actinomycetes (*8*); but shake-cultures of selected fungi had no effect on DDT (*8*).

The objectives of this study were: (i) to determine if DDT could be degraded anaerobically by soil microflora and (ii) to determine the metabolic decomposition products. The *p,p'*-DDT uniformly ring-labeled with C^{14} and carrier *p,p'*-DDT were made up in *n*-hexane so that 2 ml of standard solution, which was added to 10 g of soil (*9*), contained 0.1 mg of DDT with a C^{14} activity of 1.1 μc. After evaporation of the organic solvent, the soils were wet to 28 percent water on a dry weight basis (⅓ bar suction) and were incubated anaerobically in an atmosphere of 20 percent CO_2 and 80 percent N_2 at 30°C. Prior to extraction, the incubation chambers were flushed with N_2 and the exhaust gases were passed through two traps connected in series. The first trap (*n*-hexane) collected any loss of pesticide in the vapor phase, and the second trap (NaOH) absorbed the CO_2. Carbonate in the base was precipitated with $BaCl_2$. The resulting $BaCO_3$ was acidified, and the CO_2 evolved was collected quantitatively in 4 ml of 0.6*N* NCS (*10*). Soils were extracted at the completion of the incubation periods with a mixture of acetone, *n*-hexane, and acetic acid (50 : 50 : 1). Extracts were transferred to separatory funnels containing 300 ml of water, and the decomposition products were partitioned into the *n*-hexane layer. The *n*-hexane was dried with Na_2SO_4 and concentrated to 2 ml.

Decomposition products were separated on a two-dimensional aluminum oxide plate for the detection of DDT, DDD, DDE, kelthane, DBP, and DBM (*2*). After development, the aluminum oxide plates (impregnated with $AgNO_3$) were exposed to ultraviolet light to produce a visible dark spot. Initially, the developed thin-layer plates were exposed to x-ray film to be sure that all the activity was associated with products having predetermined R_F values from authentic samples (Table 1). Since the activity coincided with the standard compounds, the visible dark spot produced by AgCl and ultraviolet light was used to identify the products. Material from individual dark spots was transferred into counting vials containing 5 ml of counting solvent (*11*) with a suction apparatus. Both DDA and BA (*2*) were separated on an Adsorbosil-1 plate developed in a mixture of benzene, methanol, and acetic acid (50 : 8 : 2). The R_F values for DDA and BA were 0.55 and 0.48, respectively. Standard authentic samples of these two acids were spotted on each side of the soil extracts, and only the standards were sprayed with bromcresol green, which gave a yellow spot on a blue background. Material from the extracts having the same R_F values as the adjoining acids was removed by suction into counting vials. Radioactivity was determined in a liquid scintillation counter. Confirmation of the major constituents (DDT, DDD, DDE, and DDA) was made by using gas chromatography (*12*) with an electron-affinity detector.

Table 1. Two-dimensional R_F values of DDT derivatives. Layer: aluminum oxide G impregnated with $AgNO_3$ (20 mg per plate) and activated for 30 minutes at 130°C. Solvent No. 1 is *n*-heptane; solvent No. 2, a mixture of *n*-heptane, ethanol, and acetone (98:0.1:2).

Compound	R_F values in solvent	
	No. 1	No. 2
DDA and BA	0	0
Kelthane	0.02	0.11
DBP	.05	.53
DDD	.18	.45
DDT	.37	.58
DBM	.46	.65
DDE	.53	.70

DDT was dechlorinated to DDD by soil microorganisms under anaerobic conditions (Table 2). To ascertain if dechlorination was a result of microbial conversion or of catalytic conversion, a complete set of soil samples was sterilized for 1 hour in an autoclave. After each incubation period, the sterilized soil samples were plated and in all cases the soils remained sterile. DDT was not degraded in soils that were sterilized prior to incubation, which indicates that the conversion was a microbial process. After 2 weeks of incubation, 10 percent of the radioactivity in the extract was in DDD, 88 percent in DDT, and less than 2 percent in the other products. After 4 weeks of incubation, 62 percent of the radioactivity recovered was in DDD, 34 percent in DDT, and 4 percent in the other prod-

Table 2. Decomposition products of DDT isolated from soil after anaerobic incubation for 2 weeks and 4 weeks. Values, in micrograms (calculated from measurements of radioactivity), are means of two replicates.

Product	Recovery (in micrograms) after	
	2 weeks	4 weeks
DDA	0.37	0.51
BA	.24	.59
Kelthane	.15	.61
DBP	.39	.64
DDD	7.1	35
DDT	62	19
DBM	0.09	0.03
DDE	.19	.25
Total	71	57