EVIDENCE OF DIVERSITY IN BACTERIAL MERCURIC ION RESISTANCE DETERMINANTS

A report by David Weiss
on research conducted with
Dr. Nancy Hamlett as part of
Microbiology: Molecular Aspects
of Cellular Diversity.

August, 1984

tance to mercury. Resistance to mercuric ion is wide-spread among bacteria, having been described in several genera isolated from soil, sediment, and clinical environments. Research into the mechanism and genetics of mercuric ion detoxification has focused on the clinical isolates, where mercury-containing disinfectants have selected for mercury resistance. Although three resistance mechanisms have been proposed (volatilization, sequestering, and impermeability), the 100-plus studies so far conducted have found Hg^{2+} added to the growth medium collects in the head-space gas. Only a minority of these studies have reported the chemical form of the mercury, and in these that has been Hg^{0}. (Because organomercurials are also volatile, more than one mechanism for volatilization may exist.) With the Hg determinant mapped in over 800 isolates, all have been found to be on plasmids, often on transposons.

The two Hg^{R} determinants best characterized on the mechanistic and genetic level are Tn501, originally found on pVS1 in Pseudomonas aeruginosa, and R100, isolated from a Shigella. Both mer operons have closely related DNA sequences, and they specify mercuric ion reductases with the same serotype (Foster, 1983). While the relatedness of known Hg^{R} determinants is striking, so little progress has been made towards characterizing mer genes from bacteria found in non-clinical settings, that generalizations about Hg^{R} in bacteria cannot be made safely.

One of us (Hamlett) had previously isolated numerous strains

from the same locality with radioactive probes constructed from the mer genes on Tn501 and R100.

MATERIALS AND METHODS

Bacterial Strains and Plasmids. Table 1 lists the bacterial strains and plasmids used in this study along with the plasmid selective markers and source of each strain. Probes were constructed from pACYC::Tn501 and pDB7, which contains the mer genes from R100 cloned into the PstI site of pBR322. The remaining bacteria served as hybridization controls. All were maintained on LB (Miller, 1972) amended with appropriate selective agents: ampicillin 50 ug/ml, chloramphenicol 30 ug/ml, kanamycin 50 ug/ml, tetracycline 12.5 ug/ml (antibiotics were obtained from Sigma Chemical Co. and prepared as concentrated stock solutions following Maniatis et al., 1982, and stored at -20°C), and 10^{-5} M Hg^{2+}, supplied as $HgCl_2$.

Construction of Radiolabled Probes.

A) Growth of Cells. Both pACYC184::Tn501 and pDB7 were grown in selective media with vigorous shaking at 37°C until late log phase (4-6 hrs). 2 ml of these cultures were used to inoculate 400 ml non-selective LB divided between two 500 ml Erlenmyer flasks and grown as above.

B) Isolation of Plasmid DNA. Bacteria were harvested by centrifuging for 10 minutes at 7500 rpm and 20°C in a Sorval Superspeed RC2-B automatic refrigerated centrifuge equipped with a GSA rotor. The supernatant was discarded and the cells were

h at 20,000 rpm and 20°C to pellet cell debris. Plasmids were purified from the supernatant by cesium chloride density centrifugation as described by Maniatis et al., 1982, with these modifications: 1) 1.1g CsCl per ml solution was added to the supernatant; 2) Gradients were established by centrifuging 48 h at 37 rpm and 20°C in a Beckman Model L5-65 Ultracentrifuge equipped with a type 70.1 Ti rotor; 3) Plasmid bands were visualized using long-wave uv light, collected through an 18g hypodermic needle (Yale) and rebanded as before; and 4) After the second banding ethidium bromide was extracted using 1-Butanol saturated with TE (Maniatis et al., 1982) and letting the organic and aqueous phases separate 30 sec before discarding the organic (top) phase. Cesium salts were removed from the preparation by dialysis against 8 changes of TE (minimum of 2h dialysis per change) and the plasmids were concentrated by adding ammonium acetate to a molarity of 1.5 and percipitating from 2 volumes of ethanol.

C) Restriction Digests. All restriction enzymes came from New England Biolabs. EcoRI and HindIII were used to cut pACYC184::Tn501 under the following assay condition: 75 mM NaCl, 25 mM Tris-Cl (pH 7.8), 10 mM MgCl , 100 ug/ml BSA, 1 mM DTT. StuI and NcoI were used to cut pDB7 under this assay condition: 125 mM NaCl, 8 mM Tris-Cl (pH 8.0), 8 mM MgCl , 3 mM 2-mercaptoethanol, 100 ug/ml BSA. Other conditions of the digests were as specified by the supplier.

D) Elution of mer-containing Fragments. The pDB7 digest was

using E Buffer as the electrolyte (Kado and Liu, 1981). Restriction fragments were recovered by electrophoresing onto NaCl-treated Whatman Chromotography paper DE81 and later rinsed off the paper using a solution of 1 M NaCl, 10 mM Tris-Cl (pH 8.0), and 1 mM EDTA as desribed by Dretzen et al. (1981). The fragments were percipitated 2x from ammonium acetate and ethanol (as above), resuspending the pellet in 500ul TE. The fragments were quantified by ethidium bromide fluorescence, percipitated again, and suspended in a volume of sterile glass-distilled H_2O yielding a concetration of DNA convenient for nick translation.

E) The mer-fragments were labled with ^{35}S-dATP (Amersham code SJ. 304) using Bethesda Research Laboratory's nick translation kit (Cat. No. 8160SB). Labled DNA was recovered via molecular sieve chromatography through Sephadex G-50-80 (Sigma Chemical Co.) columns as described by Maniatis et al. (1982).

Preparation of Bacterial Isolates for Screening with mer Probes. 330 aerobic, chemoheterotrophic bacteria isolated by Hamlett from the Great Sippewisset Marsh, Cape Cod, MA, during June, 1981 (Hamlett, personal communication) were probed. These bacteria were isolated from experimental plots maintained scince 1970 by the Boston University Marine Program and the Woods Hole Oceanographic Institute. Control plots are untreated. High-fertilizer plots receive 50.4 g/m^2/2 weeks (late April to early November) commercial fertilizer containing sewage sludge. Extra-high-fertilizer plots, established in 1974, receive 151.2 g/m^2/2

fertilizer plots, allowing nutritional effects on the marsh biota to be distiguished from effects of contaminants in the sewage sludge fertilizer. One of those contaminants is mercury. Roughly equal numbers of isolates were selected on ZoBell medium 2216E (Rheinheimer, 1977) containing 6mg/ml $HgCl_2$, and on unamended ZoBell medium. The former collection is presumed to be a representative cross-section of the aerobic, chemoheterotrophic marsh bacteria resistant to mercuric ion, while the second group of isolates is presumed to be representative of the entire aerobic, chemoheterotrophic bacterial population of the marsh. Bacteria were streaked onto the appropriate medium from frozen cultures before transferring to nitrocellulose paper (Millipore type HA filters, cat. no. 085 25, and Schleicher & Schuell BA85 filters) in a grid pattern. The filters were prepared for hybridization as described by Davis et al. (1980)

Hybridization. Immediately prior to hybridization, DNA bound filters were washed for 30 min with mild agitation on a rotating platform shaker in 50 ml prewashing solution per filter (Maniatis et al., 1981). Filters were removed and rinsed briefly in another 50 ml prewash solution while a forceps was used to gently lift off large pieces of cell debris still clinging to the nitrocellulose paper. Hybridization procedures were modified from Davis et al. (1980). Up to 4 filters were placed in a heat-sealable plastic bag, which was then sealed using a Dazey Seal-A-Meal model 6000. 4 ml/filter of 5x SSPE, 0.3% SDS and 100 ug/ml

was resealed and let stand for 5 min. Then 10 cpm DNA probe per filter plus 100 ul TE was injected, and the bag was resealed after removing air bubbles. Filters were hybridized 14 h at 65°C. Filters were washed 4x in 50 ml per filter 1 mM $Na_{1.5}H_{1.5}PO_4$, 0.2% SDS and 1 mM Na_2EDTA at room temperature (20-25°C). Filters were air-dried 15 min. and oven-dried 30 min. at 60-80°C.

Autoradiography. Dried filters were taped to Whatman 3MM paper, overlaid directly with a sheet of Kodak XAR-5 x-ray film, loaded into Kodak x-ray exposure holders and exposed 14-39 h.

RESULTS AND DISCUSSION

Table 2 lists the isolates scoring positive in at least one of the hybridizations. All isolates were screened twice with the pDB7 mer probe and once with each of the Tn501 mer probes. Unfortunately, nearly all of the hybridizations occurred on one filter and the autoradiograph of the Tn501 2.19 kb fragment hybridization to this filter was too smudged to interpert. Hybridizations were scored positive even if fainter than negative controls (eg. HB101). Because so few of the isolates showed even this hint of hybridization, we felt it best to err on the conservative side in discounting DNA homology.

Of the 330 salt marsh isolates screened, 154 were isolated on ZoBell medium, while 176 were isolated on ZoBell medium amended with $HgCl_2$. Note that the first category includes Hg^{2+} resistant bacteria. Each of the four types of experimental plots

lizer 86.

Only 26 of the isolates, 8% of the total screened, hybridized to at least one probe. As 53% of the isolates screened have a Hg^{2+} resistant phenotype, the low rate of hybridization implies that the known Hg^R determinants are rare in Sippewissett marsh. Where hybridization did occur, it appeared considerably weaker than to positive controls, indicating that to the extent the known Hg^R determinants occur in the marsh, they have diverged evolutionarily.

The hybridizations are not as self-consistent as we would like them to be, and attempts to replicate our results would be useful. Although 19 isolates hybridized to the pDB7 mer probe during the first trial and 20 the second (each used a different batch of filters but the same batch of probe), the index of similarity was only 0.56 (= # of isolates hybridized in both trials/# hybridized in either trial.). 25 isolates hybridized to the Tn501 5.76 kb mer probe. The index of similarity between the Tn501 and second pDB7 hybridizations, chosen because these were performed concurrently, is 0.76. A higher index might be expected because the 5.76 kb Tn501 mer fragment carries part of the mercuric reductase that undergoes an immunnological cross-reaction with the reductase from pDB7. Because the plasmid pACYC184::Tn501 was isolated as open circular DNA, we were unable to separate the mer-containing restriction fragments from all the chromosomal DNA. Hybridization to negative controls appeared

implying that DNA sequences were recognized in the Tn501 probe that were not present in the pDB7 probe. These sequences could be either chromosomal DNA or parts of Tn501 not belonging to the mer operon.

→ Hybridization was more frequent among isolates from unamended than Hg^{2+}-amended ZoBell medium. 73% of the organisms which hybridized were isolated on unamended ZoBell medium. Thus 16% of the isolates obtained without the addition of Hg^{2+} hybrid-ized while §1% of the isolates selected for Hg^{2+} resistance hybridized. This result was unexpected, but can be accounted for in part by the presense of Hg^{2+} resistant isolates in the population selected on unamended ZoBell medium. However, some of the isolates which hybridized are known to have a Hg^{2+} sensitive phenotype. Other factors underlying this unexpected observation may be the presense of unexpressed (defective?) mer genes in the isolates, hybridization to non-mer DNA parts of the probe, and regions of DNA in the marsh bacteria that are homologous to the probes, but which code for different proteins (as might occur if the reading frame were shifted). Further evidence that Hg-stress is not selecting for Hg^{R} determinants similar to the known ones comes from the even distribution of hybridization-positive isolates among the different types of experimental plots: 58% of the isolates which hybridized came from control or urea phosphate plots, 42% from high fertilizer and extra-high fertilizer plots.

Although hybridization was rare, when it did occur, it was

the medium. 6 Vibrio/Aeromonas-type isolates hybridized. While this is 23% of the hybridizations, this category of isolates accounts for only 9% of the isolates screened and 7% of the Hg resistant isolates screened. The 3 hybridizing Alcaligenes comprise 12% of the hybridizing population, yet only 4% of the total isolates screened and 1% of the Hg^{2+} resistant isolates screened. Both screened Spirillum hybridized; they are §1% of the isolates obtained on unamended ZoBell medium and did not appear in attempts to isolate Hg^{2+} resistant bacteria. This is also true for the Flavobacteria, a group in which all 3 isolates hybridized. No Bacilli hybridized. Bacilli are 28% of the isolates probed, 15% of the probed isolates selected on Hg^{2+}-amended ZoBell medium. The 4 Cytophaga/Flexibacter that hybridized are 5% of those screened, but they comprise 27% of the total probed isolates, 30% of the Hg^{2+} resistant isolates. Neither of the 2 Pseudomonas/Alteromonas-type isolates that hybridized are Hg^{2+} resistant. While accounting for 8% of the isolates that hybridize, this category of isolates accounted for 30% of the total probed population, 45% of the probed population selected for Hg^{2+} resistance.

BIBLIOGRAPHY

Davis, R.W., D. Botstein, and J.R. Roth. 1980. A Manual for Genetic Engineering: Advanced Bacterial Genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Dretzen et al. 1981. Anal. Biochem. 112:295.

Rev.:47:361-409.

Kado, C. I., and S. T. Liu. 1981. Rapid procedure for detection and isolation of large and small plasmids. J. Bacteriol. 145:1365-1373.

Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Miller, J. H. 1972. Experiments in Molecular Genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Rheinheimer, G. 1977. Regional and seasonal distribution of saprophytic and coliform bacteria. p. 121-137. In G. Rheinheimer, ed. Microbial Ecology of a Brackish Water Environment. Springer-Verlag, Berlin.

Robinson, J.B., and O.H. Touvinen. Mechanisms of microbial resistance and detoxification of mercury and organomercury compounds: physiological, biochemical, and genetic analyses. Microbiol. Rev. 48:95-124.

	Strain	Plasmid	Phenotype
E. coli	HB101	None	Wild Type
	RD431	pBR322	Amp^R, Tc^R
	SK1592	pDB7	Tc^R, Hg^{2+R}
	Not Available	pACYC184	Cm^R, Tc^R
	JC5466	pACYC184::Tn501	Cm^R, Hg^{2+R}
	W1485	RP1	Amp^R, Kan^R, Tc^R
	UB1301	RP1::Tn 502	Amp^R, Kan^R, Tc^R, Hg^{2+R}
Pseudomonas	Not Available	PpG6	Oct^+, Hg^{2+R}