Association of Mercury Resistance with Antibiotic Resistance in the Gram-Negative Fecal Bacteria of Primates

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Gram-negative fecal bacteria from three longitudinal Hg exposure experiments and from two independent survey collections were examined for their carriage of the mercury resistance (*mer*) locus. The occurrence of antibiotic resistance was also assessed in both mercury-resistant (Hg^r) and mercury-susceptible (Hg^s) isolates from the same collections. The longitudinal studies involved exposure of the intestinal flora to Hg released from amalgam “silver” dental restorations in six monkeys. Hg^r strains were recovered before the installation of amalgams, and frequently these became the dominant strains while amalgams were installed. Such persistent Hg^r strains always carried the same *mer* locus throughout the experiments. In both the longitudinal and survey collections, certain *mer* loci were preferentially associated with one genus, whereas other *mer* loci were recovered from many genera. In general, strains with any *mer* locus were more likely to be multiresistant than were strains without *mer* loci; this clustering tendency was also seen for antibiotic resistance genes. However, the association of antibiotic multiresistance with *mer* loci was not random; regardless of source, certain *mer* loci occurred in highly multiresistant strains (with as many as seven antibiotic resistances), whereas other *mer* loci were found in strains without any antibiotic resistance. The majority of highly multiresistant Hg^r strains also carried genes characteristic of an integron, a novel genetic element which enables the formation of tandem arrays of antibiotic resistance genes. Hg^r strains lacking antibiotic resistance showed no evidence of integron components.

Mercury resistance (Hg^r) was the first of 12 distinct bacterial plasmid-determined metal resistance loci (including arsenic, antimony, boron, cadmium, chromium, cobalt, copper, nickel, silver, tellurium, and zinc) to be described. While many of the other metal resistance loci are found predominantly in bacteria from soil and industrial waste rather than in normal mammalian flora and pathogens, resistance to mercury compounds (*mer*) is common in both (46).

The biochemical mechanism of Hg^r in every case which has been examined is the reduction of the reactive ionic form, Hg(II) , to the less reactive, volatile, elemental form, Hg(0) (32). The plasmid-encoded *mer* locus consists of genes for the cytoplasmic Hg(II) reductase enzyme and a transport system (consisting of either two or three proteins) which brings Hg(II) into the cytoplasm for reduction by the Hg(II) reductase. The genes for the entire system are arranged as an operon under both positive and negative control of the protein MerR, an exquisitely sensitive DNA-binding protein which has a 10^4 -fold-higher affinity for Hg(II) than does mercaptoethanol (43). A subset of the naturally occurring loci which confer inorganic Hg^r also carry another enzyme, organomercurial lyase, which removes the carbon moiety from such compounds as methyl mercury and merthiolate, leaving Hg(II) , which is then a substrate for the Hg(II) reductase (3, 4). Strains carrying organomercurial lyase are thus resistant to and biotransform both inorganic and organic mercurial compounds.

The incidence of Hg^r bacteria increased in mercury-polluted soil and water (2, 21, 26) and in Japanese hospitals in a manner coincident with the use of mercurial antiseptics (37). Thus, the prevalence of Hg -metabolizing bacteria in a niche varies in response to Hg exposure in that environment. We have previ-

ously observed that resistance to Hg occurs frequently in human fecal flora and is highly correlated with the occurrence of multiple antibiotic resistance (47). We have also found that the Hg released from amalgam (“silver”) dental fillings in monkeys leads to rapid enrichment in the oral and fecal floras of many genera of bacteria resistant to Hg and antibiotics (47).

There is now concurrence that dental amalgam is the most prevalent source of mercury exposure for the general population in developed countries. Chronic inhalation and swallowing of amalgam mercury vapor are the major contributors to the total body burden of mercury in the United States population (15). The U.S. Public Health Service’s Agency for Toxic Substances and Disease Registry (1) reported recently that daily exposure to Hg from amalgam in the general population exceeds both the acute and chronic minimal risk levels for Hg allowed occupationally by 12- and 18-fold, respectively. The installation of 12 to 16 amalgam fillings in monkeys (8, 19) resulted in chronic Hg accumulations in the gingiva and intestinal contents corresponding to concentrations routinely used for the selection of Hg-resistant bacteria in laboratories (ca. 50 μM). Similar Hg concentrations have previously been found in humans with amalgam restorations (12, 36, 41). In a recent prevalence study with human subjects, Österblad et al. (36) reported that the levels of both ampicillin resistance and nalidixic acid resistance were higher in amalgam-exposed groups than they were in those who had never had amalgams ($P < 0.05$). They also showed that in every category of multiresistance, the frequency of Hg^r strains exceeded that of Hg^s strains by as much as two- to fourfold. In another recent longitudinal study with human subjects, Edlund et al. (12) showed an excess of antibiotic resistance in Hg^r strains of certain fecal genera from amalgam bearers compared to that of amalgam-free controls.

Since Hg^r loci and antibiotic resistance are commonly found on the same plasmids and transposons (18, 40, 46), chronic

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TABLE 1. Sources of bacterial isolates and strains^a

mer locus	No. of isolates or strains									
	Human			Monkey					Other ^b	Total
	EPS	ECOR	Total	PA			ECOR	Total		
				16 and 95	830 and 980	78 and 79				
1	10, 10	1, 1	11, 11		4, 3	2, 2	1, 1	7, 6	2, 2	20, 19
2	5, 5		5, 5		22, 5	2, 1		24, 6		29, 11
3	10, 10		10, 10			9, 2		9, 2		19, 12
4	21, 21		21, 21	12, 3	44, 27	12, 8		68, 38	1, 1	90, 60
5/6	1, 1		1, 1	3, 2		15, 6		18, 8		19, 9
9	2, 2		2, 2							2, 2
10	5, 5		5, 5							5, 5
Undefinable	5, 5	1, 1	6, 6			3, 1	1, 1	4, 2		10, 8
Not tested					47 ^c			47 ^c		47 ^c
Subtotal	59, 59	2, 2	61, 61	15, 5	117, 35	43, 20	2, 2	177, 62	3, 3	241, 126
None (Hg ^s)	22, 22	1, 1	23, 23	1, 1	111, 30	93, 37	9, 9	214, 77		237, 100
Total	81, 81	3, 3	84, 84	16, 6	228, 65	136, 57	11, 11	391, 139	3, 3	478, 226

^a See Materials and Methods for the distinction between isolate and strain as used in this work. The number of isolates is in standard typeface; the number of strains observed within a group of isolates is in boldface.

^b Two Hg^r strains in the ECOR collection were from dogs, and a third Hg^r strain was from a leopard.

^c The mer loci in these 47 isolates were not characterized at the molecular level because they are presumptive siblings (on the basis of their biotypes, ERIC types, and resistotypes) of strains whose mer loci were isolated from the same animal and were characterized in detail. They are included in the data for Fig. 4 and 5, in which the type of mer locus is not an issue. They are not included in the data for Fig. 6 since we did not actually determine their molecular mer types. There are nine distinct strains in this group, but they are indistinguishable from others enumerated above and are not indicated separately here.

exposure to Hg from amalgams may foster (via coselection of linked markers) the persistence of such multiply resistant strains even in the absence of frequent antibiotic use. As part of a longer-term study to evaluate this hypothesis, we have recently devised molecular genetic tools to define 10 structurally distinct mer loci (29). The goals of the work reported here were to discover the generalizability of a previously suggested genus preference of mer loci (28) and to ask whether the association of the Hg^r phenotype with multiple antibiotic resistances, previously observed in bulk fecal cultures (47), was also a property of individual strains isolated from those cultures.

MATERIALS AND METHODS

Bacterial isolates and strains. We examined three distinct collections of the family *Enterobacteriaceae* (Table 1). The Primate Amalgam (PA) collection consists of individual gram-negative fecal bacteria collected during our previous (1991 to 1993) study (47), comprised of three independent experiments of 7, 19, and 23 weeks (Fig. 1). As previously described (47), each experiment used two adult monkeys, into whose teeth were installed 16 small occlusal surface dental amalgam fillings. For each animal, duplicate inocula of freshly voided fecal material were taken on sterile swabs twice weekly. Each swab was used to inoculate duplicate nonselective master plates, which were streaked in three directions for the isolation of single colonies. Master plates were replica plated onto a series of test plates containing 100 µM HgCl₂, 15 µg of tetracycline per ml, or 50 µg of ampicillin per ml and a final nonselective plate. At each sampling time, individual isolates were selected from the most dilute area of each plate (i.e., representing the strain most abundant on that particular plate). After

restreaking a clone to confirm its phenotype, each bacterial isolate was stored at -70°C in cell freezing medium (0.5% yeast extract, 5% dimethyl sulfoxide, 5% glycerol, 0.08 M sodium phosphate [pH 7.2]). In all experiments, samples were taken before and during the placement of amalgams; in the two longer experiments, animal feces were sampled after amalgams had been replaced with non-metallic restorations. Thus, each animal served as its own longitudinal control.

The ECOR collection is a standard reference collection used in *Escherichia coli* population biology studies (34). Here, we examined the 17 ECOR strains which were either Hg^r (*n* = 7) or Hg^s and isolated from humans or nonhuman primates (*n* = 10). The Environmental Plasmid Survey (EPS) collection consists of fecal strains isolated from humans in the late 1970's (14, 28). These two latter collections were stored as described above for the PA collection; our copy of the ECOR collection was received from Dan Dykhuizen in 1986.

In the analysis presented here, the term isolate refers to a pure clone derived from a fecal sample; each such isolate in the PA and EPS collections was given a unique serial number. The ECOR strains already had specific designations. To classify isolates as strains, we first biotyped and resistotyped them (see below) and then used arbitrarily primed PCR (AP-PCR) fingerprinting with enterobacterial repetitive intergenic consensus (ERIC) primers (49) (see below) on members of the same biotype to define specific ERIC types. Two isolates having the same biotype were considered distinct strains if they differed in either the ERIC fingerprint or the resistotype. Moreover, as a conservative measure, isolates derived from distinct animals or humans were considered distinct strains even if they had the same biotype, resistotype, and ERIC type. For each collection we examined, the numbers of isolates and strains recovered, as defined here, are listed in Table 1. The 478 isolates we describe yielded 226 distinct strains.

Biotypes and resistotypes. Bacterial cultures were taken from storage at -70°C, inoculated onto Luria agar, and incubated at 37°C overnight. Isolates were biotyped by using the API 20E system from bioMérieux Vitek, Inc. (Hazelwood, Mo.). Resistotypes were determined by replica plating two colonies of each isolate. The test concentrations of the compounds used were as follows:

FIG. 1. Experimental sampling time lines of fecal swabs collected from primates for the three amalgam installation experiments from which the PA collection was derived. Amalgam filling replacements with glass ionomer fillings were done only in experiments 2 and 3. For sampling procedure details, see Materials and Methods.

HgCl₂, 100 µM; phenylmercuric acetate, 25 µM; ampicillin, 50 µg per ml; chloramphenicol, 25 µg per ml; kanamycin, 25 µg per ml; nalidixic acid, 50 µg per ml; streptomycin, 25 µg per ml; sulfadiazine, 500 µg per ml; and tetracycline, 15 µg per ml. All compounds were tested in Luria agar, except for sulfadiazine, for which Mueller-Hinton agar was used. In enumerating resistances here, phenylmercuric acetate resistance was not counted separately from Hg resistance. Sensitive and resistant laboratory standard strains were included on replica master plates to monitor the potencies of selective agents.

Bacterial strain DNA fingerprinting. Primers based on the ERIC sequence (49) were used for fingerprinting each isolate (see Fig. 2). Reactions were carried out in a total volume of 50 µl containing 50 pmol of each primer, 0.12 mM (each) deoxynucleoside triphosphates, 1.5 mM MgCl₂, 2.5 U of *Taq* DNA polymerase, and 1× PCR buffer supplied with the enzyme. Template DNA was obtained from whole cells picked directly from overnight plates. A sterile toothpick was touched to the culture and used to inoculate 15 µl of nuclease-free deionized H₂O in sample tubes. One wax bead was added, and samples were heated to 95°C for 5 min and then cooled to 25°C for 1 min. The remaining reagents were added on top of the wax layer. Reaction mixtures were cycled by the method of Herrick et al. (22). Ten microliters of each reaction mix was electrophoresed on 2% agarose gels in 1× TBE (90 mM Tris base, 90 mM boric acid, 2 mM EDTA [pH 8.0]); gels were stained with 0.1 mg of ethidium bromide per ml and photographed. Each distinct ERIC amplicand pattern was given a specific ERIC type designation consisting of a letter designating the genus (e.g., E for *Escherichia* spp.) and a number specifying the pattern (e.g., E1) which distinguished it from other *Escherichia* fingerprints. Isolates of a given ERIC type often had similar but not always identical API 20E biotypes.

Typing *mer* loci. The loci conferring mercury resistance in each strain were characterized by Southern hybridization and PCR amplification with primers and probes based on published DNA sequences of *mer* operons (29). Note that locus 4 was separated into distinct subtypes, a, b, c, and d, depending on the state of a polymorphic position in the *merT* gene (29). Although it is interesting for other reasons (50a), this distinction is not relevant for the observations reported here and for simplicity's sake has not been included.

Conjugation. All matings were carried out on the surface of a nonselective (i.e., Luria broth) agar plate at 37°C for 24 h. Donors were various natural isolates carrying Hg^r and generally one or more antibiotic resistances. The following two types of recipients were used: standard restriction modification-defective laboratory strains (e.g., C600) and natural Hg^s isolates of the same genus as that of the Hg^r donor strain. The standard laboratory recipients were resistant to both nalidixic acid and rifampin; Nx^r and R^r derivatives of each natural Hg^s isolate were derived in the laboratory for use in mating experiments. Postmating selection was carried out by streaking each mating mixture onto Luria broth agar containing two appropriate selective agents. At least five resulting colonies from each mating were then screened by replica plating for the presence of additional nonselected markers. The ERIC fingerprints of putative exconjugants were compared with those of the donor and recipient to confirm that conjugation had taken place. The type of *mer* locus transferred to each exconjugant was discerned via PCR, restriction digestion, and Southern hybridization as previously described (29).

Detection of integron components by PCR. Oligonucleotide primers 5'CS, 3'CS, IntI-1 (Bam660), and IntI-2 (Pvu900) (27) were synthesized by the University of Georgia Molecular Genetics Instrumentation Facility, Athens. The IntI-1 and IntI-2 primers were used to amplify the integrase gene (*intI*) region of the Tn21-like transposon. PCR primers 5'CS and 3'CS were used to amplify the 5' and 3' conserved segments, respectively, of the integron with integrated antibiotic resistance gene(s). A standard PCR protocol with whole-cell lysates and a hot start method was employed (50). Amplification reactions were carried out in a total of 75 µl containing 25 pmol of each primer, 0.25 mM (each) deoxynucleoside triphosphates, 1.5 mM MgCl₂, 2.25 U of *Taq* DNA polymerase, and 1× PCR buffer supplied with the enzyme. For templates, cells grown overnight were picked from plates by touching growth with a sterile toothpick and transferring it into 25 µl of nuclease-free deionized H₂O. A wax bead reaction was prepared as above. For the IntI-1 and IntI-2 primers, the PCR cycle was 94°C for 30 s, 58°C for 30 s, and 72°C for 1 min for 30 cycles and then held at 15°C. For the 5'CS and 3'CS primers, the PCR cycle was 94°C for 1 min, 50°C for 1 min, and 72°C for 5 min with an additional 5 s per cycle for 35 cycles and then held at 15°C. PCR products were electrophoresed, visualized, and recorded as described above. Appropriate positive and negative controls were included in every PCR run.

RESULTS

Definition of the molecular types of Hg resistance loci observed in these strain collections. To date, six nearly complete sequences of *mer* operons from gram-negative bacteria have been reported (cited in references 29 and 35). In addition, there are partial sequences of the *merR-merOP* regions of 10 more strains (35). We expected to find examples of many of these standard *mer* loci among the natural isolates described here and to observe loci distinct from those whose sequences

are currently available. In examining these wild isolates and previously described *mer* loci, we have recently defined 10 gram-negative *mer* loci (referred to as locus 1 through locus 10) distinguishable by a set of phylogenetic character states, including the presence or absence of certain genes and restriction site polymorphisms of those genes (29). All but four of the *mer* loci (loci 3, 5, 9, and 10) recovered from primate flora are clearly related to those recovered by others from environmental and clinical sites. We found no occurrences among primate intestinal strains of the standard sequenced locus 7, which was originally obtained from *Thiobacillus* sp. We also found only deleted variants of locus 8, which confers resistance to organomercurials such as phenylmercuric acetate. These abbreviated broad-spectrum resistance loci (locus Δ8) were found only with another intact locus (typically locus 4) and are not enumerated separately in this report.

Association of specific *mer* loci with individual strains of fecal bacteria prior to, during, and after the installation of amalgam dental restorations. We collected fecal bacteria from three distinct, sequential experiments (47), each involving a pair of adult monkeys, individually caged in a room separate from the rest of the vivarium. We recovered 175 Hg^r and 205 Hg^s isolates in the PA collection. The distributions of biotypes among Hg^r and Hg^s strains were similar. The ERIC AP-PCR fingerprinting technique applied to individual isolates allowed us to identify (Fig. 2) and to follow the occurrence over time (Fig. 3) of distinct bacteria, each of which was given a name consisting of a letter denoting the genus (determined with an API 20E; see Materials and Methods) and a number denoting the unique pattern of PCR products generated from its DNA with the ERIC primers (Fig. 2 and 3) (see Materials and Methods). The ERIC AP-PCR technique was sufficiently robust that we were able to track repeated isolations of the same ERIC type from a given animal and to observe its occurrence in other animals in subsequent experiments and even in other collections (for example, ERIC types E1, E2, E3, and E13) (Fig. 2). The ERIC primer technique has previously been shown to work well with fermentative members of the *Enterobacteriaceae* family and pseudomonads (10, 23, 25, 30, 49). We also found that it allowed consistent distinctions among isolates of these bacterial families recovered in monkey experiments as well as from humans in the EPS collection (Fig. 3 and data not shown). Several ERIC types, for example, E1 and E16, occurred in both Hg- and/or antibiotic-resistant strains and in Hg- and/or antibiotic-susceptible strains (Fig. 3). However, there were no apparent changes in ERIC fingerprint patterns (Fig. 2) as a function of the resistances carried by these independent isolates.

First experiment: cynomolgus monkeys 16 and 95. A relatively small number of Hg^r isolates was saved from monkeys 16 and 95, the first pair we studied. The 12 enterobacterial isolates from monkey 95, all *E. coli* and *Leclercia* isolates, carried *mer* locus 4. The *Pseudomonas* ERIC type recovered from monkey 16 carried two *mer* loci, the novel locus 5 and locus 6, which is indistinguishable by the measures we used from Tn501 (29). All ERIC types collected from these first two animals were distinct from those found in the subsequent two pairs of monkeys, with the exception of Hg^s *Pseudomonas* ERIC type P1, which was found in monkey 16 (not shown) and later found in one of the last pair of animals examined, monkey F78, when its amalgams were in place (Fig. 3).

Second and third experiments: rhesus monkeys L980 and R830 and cynomolgus monkeys F78 and F79, respectively. The last two experiments ran for 19 and 23 weeks, respectively, and afforded a richer picture of the ecological succession of susceptible and resistant strains under the impact of amalgam Hg

FIG. 2. ERIC fingerprints of some *E. coli* strains from the PA and ECOR collections. Bracketed lanes indicate isolates with identical fingerprints isolated from different collections (in the case of E1, they were isolated from two monkeys). In each designation, the letter denotes the genus and the number denotes the ERIC pattern (see Materials and Methods and the legend for Fig. 3).

exposure (Fig. 3). From monkeys L980 and R830, 228 isolates (117 Hg^r and 111 Hg^s) were recovered. Monkeys F78 and F79 yielded 136 isolates (43 Hg^r and 93 Hg^s). Note that Hg-susceptible strains were recoverable even when fecal Hg concentrations reached the millimolar level during the week immediately after amalgams were installed (47). One Hg^s strain recovered from monkey F78 gave PCR products that were consistent with the fact that it carried a partially deleted *mer* operon, which was most closely related to locus 1 (ERIC type E4) (Fig. 3).

Although each experiment showed increases in the number of Hg^r bacteria when amalgam fillings were in place, the succession of specific strains and *mer* loci over time in the last two experiments (Fig. 3) also revealed the following.

(i) Each animal had one or more idiosyncratic strains which persisted through more than one period. For example, Hg^s ERIC type E12 occurred before and after amalgam placement in monkey L980 but was not recovered in other animals.

(ii) Several strains were especially widely dispersed, occurring in more than one monkey and even in different experiments. Hg^s ERIC type E1 was recovered 25 times in all four animals during one or more periods as follows: L980, 8 isolates; R830, 14 isolates; F78, 1 isolate; and F79, 2 isolates. E1 was also recovered as a Hg^r (locus 4) variant in monkey R830. *Citrobacter* C6 was recovered only in Hg^r form (with locus 4), but it occurred before and during amalgam placement in L980, during amalgam placement in R830, and before and during amalgam placement in F79. *Citrobacter* C11 (with locus 2) was also recovered from two distinct periods in monkeys L980 and R830. Finally, *Leclercia* L1 (with locus 4) also occurred in all four animals during (and before in F78) amalgam placement.

(iii) In general, a strain with a given ERIC type might be

either Hg^r or Hg^s; however, when the strain was Hg^r, its ERIC type was always associated with the same *mer* locus, though not necessarily the same set of antibiotic resistances. For example, ERIC type E1 occurred in its Hg^r forms (always carrying locus 4) only in monkey R830, where it had three distinct (but overlapping) combinations of antibiotic resistances. When it was isolated from monkey R830 with an Hg^r phenotype, ERIC type E1 was Ap^r Cm^r Sm^r Su^r Tc^r (isolate 388H), Ap^r Cm^r Km^r Sm^r Tc^r (isolate 400H), or Ap^r Cm^r Sm^r Tc^r (isolate 413H). In its Hg^s occurrences, E1 manifested all of the antibiotic resistance patterns seen in these three Hg^r variants, as well as a pattern not seen in them, Ap^r Sm^r Su^r Tc^r (e.g., isolate 405T). ERIC type E16 occurred in both Hg^s and Hg^r forms in monkeys R830 and L980. As a Hg^s ERIC type, E16 was isolated five times from monkey L980 and nine times from monkey R830; in no case did it have any of the antibiotic resistances for which we screened. When it was Hg^r, E16 always had locus 4 and streptomycin resistance; on two occasions, E16 also had Pm^r (isolates 362H and 366H). The monkey pairs themselves also differed in the strains they carried. In monkeys R830 and L980, ERIC types E1, E15, and E16 were recovered as both Hg^r and Hg^s isolates (Fig. 3). In contrast, the Hg^r ERIC types in monkeys F78 and F79 were always distinct from the Hg^s ERIC types, although one of the Hg^s ERIC types in this pair was the peripatetic E1, which had carried *mer* locus 4 in monkey R830 (Fig. 3).

(iv) In three of the six monkeys, Hg^r *Pseudomonas* strains became the major proportion of the isolates recovered during or after fillings were in place (monkey 16, *Pseudomonas aeruginosa* P5 [data not shown]; monkey F78 [Fig. 3], *P. paucimobilis* [also known as *Sphingomonas paucimobilis*] P1 and *P. maltophilia* [also known as *Stenotrophomonas maltophilia*] P2; and

FIG. 3. Population successions in four amalgam-exposed monkeys. For each animal, the bacterial ERIC types recovered before amalgam placement, while amalgams were in place, and after amalgams were replaced with nonmetallic restorations are indicated by ovals. Within each oval, the letter designates the specific genus (as determined by API 20E biotyping) and the associated number designates the specific ERIC fingerprint type. The genus designations are as follows: A, assorted, including spp. from *Acinetobacter*, *Proteus*, and other genera; C, *Citrobacter* and *Enterobacter* spp.; E, *Escherichia* spp.; K, *Klebsiella* spp.; L, *Leclercia* spp.; P, *Pseudomonas* and *Stenotrophomonas* spp.; S, *Serratia* spp.; V, *Khuyvera* spp.; UN, isolates which were either untypeable by API 20E biotyping or yielded no fingerprint with the ERIC primers. The strain designated E/K typed as either an *Escherichia* or *Klebsiella* sp. by API 20E biotyping and did not give a fingerprint with the ERIC primers. Ovals with thick borders indicate Hg⁺ strains, and the Hg⁺ locus found in each Hg⁺ strain is listed below the line in boldface. Ovals with thin borders indicate Hg⁻ strains. The only Hg⁺ *Escherichia* strain, E4, which carries a vestigial locus 1, is indicated by a dashed border. Locus Δ8 in strain K1 (monkey F78 before amalgam placement) confers organomercurial resistance (29).

monkey F79 [Fig. 3], *P. maltophilia* P2). Indeed, in monkeys 16 and F78, the respective *Pseudomonas* ERIC types were the only Hg⁺ strains recovered after amalgam replacement.

Occurrence of *mer* loci in two *Enterobacteriaceae* survey collections. (i) **The EPS collection.** Since each of the EPS isolates considered here was recovered from a different person and had distinct antibiotic resistance patterns, each was counted as a single strain. Fifty-four of the 59 Hg⁺ EPS strains (92%) characterized had identifiable single *mer* loci, the majority of which (46 strains = 85%) was four *mer* loci (loci 1 through 4). The genera observed in this collection included *Escherichia*, *Citrobacter*, *Enterobacter*, *Klebsiella*, *Pseudomonas*, *Proteus*, *Serratia*, and *Shigella*; all genera but *Shigella* were also found in the monkey samples discussed above. ERIC analysis was done only with the *E. coli* strains in this collection; of the 41 fingerprints found, two (E7 and E11) also occurred in the PA collection; none of the ERIC types of the EPS collection were found in the ECOR collection. Both E7 and E11 were Hg⁺ in the EPS

collection, where E7 was also Sm^r Tc^r and E11 was Ap^r Sm^r Tc^r. In contrast, when they occurred in monkeys, E7 (in F78) and E11 (in R830) were Hg⁺ and only the former carried any antibiotic resistances (Ap^r Sm^r Tc^r).

Of the 22 Hg⁺ human strains from the EPS collection, 11 were *E. coli* strains. Only two of them (E32 and E33) had ERIC patterns similar to that of any Hg⁺ EPS strain; the rest were unique. ERIC type E32 was recovered either as only Hg⁺ (isolate 1532) or as Hg⁺ and carrying Ap^r Sm^r Su^r Tc^r (isolate 1478B). ERIC type E33 was recovered as a Hg⁺ Ap^r Cm^r Sm^r Su^r Tc^r (isolate 1349B), as a Hg⁺ Ap^r resistant isolate (isolate 347), and as a strain which had no resistances (isolate 1348A) but had been initially recovered as a Hg⁺ strain in 1979.

(ii) **ECOR collection.** The Hg⁺ strains ECOR3 (from a dog), ECOR34 (from a dog), ECOR37 (from a monkey), and ECOR48 (from a human) have *mer* locus 1 (29), and the Hg⁺ strain ECOR31 (from a leopard) has *mer* locus 4. We found strains ECOR41 (from a human) and ECOR65 (from a hu-

man; previously described as Hg^s but with positive *mer* hybridization [14]) to be actually Hg^r but were not able to amplify sufficient *mer*-specific PCR products to characterize them further; they are each enumerated as undefinable in Table 1.

In addition to ERIC typing, the 7 Hg^r ECOR strains mentioned above, we examined 10 Hg^s ECOR strains (ECOR7, -18, -19, -46, -52, -54, -57, -66, -69, and -70) which had been isolated from humans and nonhuman primates (34). ERIC AP-PCR fingerprints of these 17 *E. coli* strains revealed 15 fingerprints, three of which were also found among PA strains (ECOR3, E2; ECOR70, E3; both ECOR18 and ECOR19, E13) (Fig. 2). Ten of these 17 strains were typed by Milkman by multilocus enzyme electrophoresis (31); he also found them to be distinct from each other. Only 1 of 7 Hg^r strains and 4 of 10 Hg^s strains carried any antibiotic resistances when they were recovered from storage in 1993.

Distribution of *mer* loci among various gram-negative host strains. One notable trend appeared in the distribution of *mer* loci among the gram-negative bacteria from all three collections. Loci 5, 6 (equivalent to Tn501), and 9 were recovered exclusively from pseudomonads, never from fermentative enterobacteria. Since Tn501 (the index example of locus 6) functions well in *E. coli* (7), its limitation to nonfermentative gram-negative bacteria in these collections may arise from its association with certain plasmids rather than from physiological constraints on the expression of its Hg resistance phenotype. We have previously observed (44–46) that metal resistances are associated with large, low-copy-number plasmids of limited host range in members of the *Pseudomonaceae* family.

The opposite tendency was seen in the host strain associations of the other five *mer* loci (loci 1, 2, 3, 4, and 10) we observed here. Although the number of distinct host biotypes ranged from a low of four for locus 1 (*Citrobacter*, *Escherichia*, *Kluyvera*, and *Enterobacter* strains) to a high of seven for locus 4 (*Citrobacter*, *Enterobacter*, *Escherichia*, *Klebsiella*, *Kluyvera*, *Leclercia*, and *Proteus* strains), only locus 10 appeared in both fermentative (*Enterobacter* and *Escherichia*) and nonfermentative (*Pseudomonas*) strains. Since Tn21 (the index example of locus 1) is able to express Hg resistance in both *Pseudomonas* and *Acinetobacter* strains (13) in the laboratory, this natural distribution of locus 1 may also be influenced by its association with specific plasmids whose host range is constrained to fermentative enterobacteria (e.g., R100, the IncFII plasmid which carries Tn21) (11).

In none of the three collections examined was any distinct *mer* locus exclusively associated with a single strain or biotype. With the exception of *Leclercia* strains, which were recovered only from monkeys and were always Hg^r (carrying locus 4), and pseudomonads, which (with two exceptions) were always Hg^r in either monkeys or humans, Hg^r variants of all biotypes were found as frequently as were Hg^s variants of the same biotypes.

Vestigial *mer* loci. In the PA collection, we found one Hg^s ERIC type (E4; three distinct isolates over a 2-week period from monkey F78) which yielded PCR products that hybridized with *mer* probes (i.e., were bona fide *mer* PCR amplicands). The *merA* PCR product of this Hg^s ERIC type most closely resembled locus 1 (equivalent to Tn21). Two of the Hg^s EPS strains also gave rise to bona fide *mer* PCR products, but they have not been examined further. It appears that vestigial *mer* loci do occur, albeit rarely, in Hg^s strains. As noted above, *mer* loci conferring broad-spectrum resistance were found only as internal deletants of locus 8; it appeared that they had lost most of the *mer* genes between the promoter and the *merB* (organomercurial lyase) gene, and all were found in strains with another intact *mer* locus (29).

FIG. 4. Occurrences of antibiotic resistance phenotypes in human and monkey Hg^r and Hg^s strains. Above each bar is the actual number of primate strains recovered which carried the indicated marker. The vertical axis indicates the percentage of strains in which the indicated marker occurred; the sum of these percentages is more than 100 since many strains had more than one marker. For example, 57 of 123 Hg^r strains were ampicillin resistant; thus, ampicillin resistance occurred in 46% of Hg^r strains.

Frequencies of antibiotic resistances in Hg^r and Hg^s strains.

The six antibiotic resistance phenotypes we assessed did not occur with equal frequencies (Fig. 4). Regardless of whether strains were Hg^s or Hg^r, resistances to ampicillin, streptomycin, and tetracycline were more abundant than were resistances to chloramphenicol, kanamycin, and sulfadiazine. However, a larger proportion of Hg^r strains had resistance to any one antibiotic than did Hg^s strains (e.g., ampicillin resistance was found in nearly 50% of Hg^r strains but in just over 30% of Hg^s strains).

We examined more closely this propensity to have ampicillin, streptomycin, or tetracycline resistance by determining the number of additional resistances in strains which already had resistance to a given agent (Fig. 5). For example, strains resistant to ampicillin had an average of 2.8 other resistances (including those to other antibiotics and Hg), whereas monkey and human strains susceptible to ampicillin had on average only 1.2 additional resistances. As resistance is a discrete (integral) variable occurring over a limited range (0 to 7 per strain here), the distributions were broad and standard deviations were large. However, the trend for strains resistant to an agent to have more resistances than strains susceptible to that agent held for all three of the most prevalent antibiotic resistance loci and for Hg^r and Hg^s. This clustering was also true for two of the more rare resistances, Cm^r and Km^r, which appeared only in strains with two to six additional antibiotic resistances. In other words, in these collections, a strain with any resistance at all (to an antibiotic or Hg) was likely to be multiresistant. A more detailed examination of Hg^r strains revealed another surprising aspect of resistance clustering.

Nonrandom association of multiresistance with *mer* loci. In the PA and EPS collections, certain *mer* loci were more likely to be found in strains with higher numbers of antibiotic resistances (Fig. 6). Specifically, there were consistent differences in the average number of antibiotic resistances per strain for the *mer* loci most often observed, loci 1 through 6, which together accounted for 169 of the 180 Hg^r isolates whose *mer* loci we

FIG. 5. Clustering of resistance loci within bacterial strains. R, resistant; S, susceptible. T-bars indicate the positive values of standard deviations.

defined (94%). Loci 5 and 6 occurred only in pseudomonads and are enumerated together here. Loci 9 and 10 were represented by two and five isolates, respectively, in only the EPS collection, and eight isolates carried untypeable *mer* loci. Isolates carrying locus 1 averaged nearly threefold more antibiotic resistances than did locus 2 isolates. Isolates with locus 3 or 4 were intermediate in their antibiotic resistances. Loci 5 and 6 occurred exclusively in pseudomonads, which were the most multiresistant strains we observed in these collections (regardless of source). Once again, the standard deviations of these averages were large (regardless of sample size). However, statistical tests (coefficient of deviation and chi-square [42]) have indicated that the occurrences of other resistances are normally distributed in all *mer* loci.

FIG. 6. Average numbers of antibiotic resistances observed in strains and isolates carrying different *mer* loci. T-bars indicate sample standard deviations; the numbers of strains and isolates in each locus are listed above each bar. Loci 9 and 10 are not included here because they were recovered only from humans and in limited numbers (two and five isolates and/or strains, respectively).

TABLE 2. Conjugative transfer of Hg^r and other resistances by strains carrying various *mer* loci

<i>mer</i> locus	No. of donor strains tried ^a	No. of donor strains transferring any resistance	Resistance(s) transferred ^b
1	3	1	Hg Ap Sm Su
2	2	2	Ap, Pm
3	3	1	Sm
4	16	10	Hg Ap Km Sm Su, Hg Ap Km, Hg Sm Su, Hg Ap Cm Sm Tc, Ap Cm Sm Tc, Ap Sm Su, Sm Su, Ap, Km, Sm
5	1	1	Hg Ap Pm Sm, Hg Pm Sm
6	3	0	None
9	1	0	None
10	1	1	Hg Pm

^a From one to eight independent isolates of various strains were tried as donors.

^b Underlining indicates that the markers were cotransferred by selection for any one of the resistances. Ap, ampicillin; Sm, streptomycin; Su, sulfadiazine; Pm, phenylmercuric acetate; Km, kanamycin; Cm, chloramphenicol; Tc, tetracycline;

Molecular genetic basis for clustering of metal and antibiotic resistances in primate enterobacteria: plasmid carriage of mercury and antibiotic resistance markers. Both antibiotic and metal resistances can occur on conjugative plasmids (as well as on the chromosome), and there have been many instances of both types of resistance being found on the same plasmid (16, 24, 33, 38, 52). One simple measure of plasmid-based genetic linkage is the cotransfer of an unselected marker. Using both laboratory strains and derivatives of antibiotic- and Hg-sensitive natural isolates as recipients, we found that most Hg^r strains transferred Hg^r-linked arrays of antibiotic resistance markers (Table 2), consistent with the *mer* locus residing on one or more plasmids in these strains.

Most of the classically described transposable elements carry just one resistance marker. Well-studied examples include Tn3, Tn5, Tn9, Tn10, and Tn501, which carry ampicillin, kanamycin, chloramphenicol, tetracycline, and mercury resistances, respectively. However, the widely found members of the Tn21 transposon family have arisen from repeated insertions of a novel transposable element, called an integron, into an ancestor of Tn2613 (6, 48) (Fig. 7). This integron is unusual in providing not just resistance to a single antibiotic but an insertion site (*attI*) and an integrase gene (*intI*) whose product (IntI) enables the formation of multiple tandem arrays of diverse antibiotic resistance genes (called cassettes) at the insertion point. At present, some 36 distinct antibiotic resistance gene cassettes have been identified in members of the family *Enterobacteriaceae* and of the genus *Pseudomonas* (20, 39). The members of the Tn21 family which result from these various coinTEGRATION events are among the most widely found transposable elements in facultative gram-negative bacteria (5, 6, 9, 51).

By PCR, we examined the strains carrying the most abundant *mer* loci for their carriage of the following integron-related elements: (i) the *intI* gene itself and (ii) inserts of antibiotic resistance cassettes. Amplification with primers IntI-1 and IntI-2 (Fig. 7) yielded a 280-bp product when a class I integron gene was present in the strain. Amplification with primers 3'CS and 5'CS yielded products of various sizes, depending on how many cassettes were inserted adjacent to the integrase gene. In both human and animal isolates, locus 2 and

FIG. 7. Composite transposon Tn21. The *tnpA* and *tnpR* genes encode functions for the transposition of Tn21 itself. The *tni* genes are part of an operon (most of which has suffered deletion in Tn21) which effects transposition of the integron element. The 3' conserved segment (3'CS) includes a gene for sulfadiazine resistance (*sulI*) and a partly deleted gene for resistance to quaternary ammonium disinfectants (*qacEA1*). The antibiotic resistance gene cassette in Tn21 carries *aadA1* (the streptomycin adenylyl transferase gene). The 5' conserved segment (5'CS) includes the integrase gene (*intI1*), which is responsible for the insertion of antibiotic resistance cassettes. Arrows indicate the positions of PCR primers designed to detect (i) whether there is anything inserted at the insertion point (primers 3'CS and 5'CS) and (ii) whether a class I integrase gene is present (primers IntI-1 and IntI-2 [I1 and I2, respectively]).

locus 3 strains were devoid of integron components by these tests (Table 3). However, in both groups, many locus 1 (75% overall) and locus 4 (50% overall) strains carried one or both integron elements. Evidence of cassette insertions was more apparent than was evidence of the integrase gene itself; this may have been due to the relative conservation of the regions for which the primers were designed (27).

DISCUSSION

Although the discovery that bacterial plasmids carry metal resistances was coeval with the discovery that they carry antibiotic resistances (40), subsequent studies of these two classes of resistance markers have largely been carried out completely independently. Microbial ecologists have concerned themselves exclusively with metal resistances as markers of environmental pollution, and clinical microbiologists have looked only at antibiotic resistances. Fortunately, this insularity is beginning to give way because of frustration at our inability to control the spread of multiresistant bacteria in nonhospitalized persons and an increasing awareness that environmental factors other than antibiotics may also influence the composition of normal flora.

As noted above, our first goal in this long-term study was to devise tools for rigorous assessment of the occurrences of Hg^r determinants (29) in the intestinal flora during a specific, quantifiable environmental stress, exposure to acute and chronic Hg intoxication. Having devised such tools, we have used them to examine the generalizability of a previously suggested genus preference of *mer* loci (28) and to ask whether the association of the Hg^r phenotype with multiple antibiotic resistances, pre-

viously observed in bulk fecal cultures (47), was also a property of individual strains isolated from those cultures. This latter point has particular clinical relevance, as a strain with a single antibiotic resistance gene is more susceptible to successful treatment (i.e., there are potentially more fallback antibiotic choices) than is one with several antibiotic resistance genes.

We found the answers to the questions mentioned above and learned some unexpected things about the *mer* locus and antibiotic resistances in the process. With respect to our first query, we found in our longitudinal studies of monkeys with amalgam Hg exposure that both preexisting Hg-resistant strains and apparently novel strains (presumably from food or other animals, including humans, in the vivarium environment) could become dominant members of the fecal facultative gram-negative flora, depending on the individual monkey examined. Thus, even in these relatively well-controlled laboratory animal microcosms, one cannot predict simply on the basis of the preexisting flora precisely which strains will survive Hg exposure and dominate during or after amalgam exposure. Our hypothesis is that the acute Hg exposure immediately after amalgam installation (47) produces considerable upheaval in the total bowel flora composition. In this model, the high postinstallation Hg concentration acts like a therapeutic course of antibiotic in perturbing the endogenous susceptible microbiota and making this niche susceptible to transient colonization by nonautochthonous strains. We have some preliminary evidence to support this hypothesis and are pursuing additional studies in this regard.

With respect to our second query, there are *mer* loci which are narrowly distributed and others which are found more widely. Loci 5 and 6 (Tn501) and 9 are found exclusively among members of the *Pseudomonaceae* family. Locus 1 (Tn21) is the next most narrowly distributed, occurring predominantly in *Escherichia* strains. The remaining loci were observed in a great variety of fermentative enterobacteria, and locus 10 occurred in both fermentative and nonfermentative enterobacteria. Locus 7 (29), which has been sequenced by others (17), did not occur in any of these strains from primate feces. Thus, rather than a simplistic genus preference, these observations suggest a range of habits, from a limited to a highly promiscuous host range, for various *mer* loci, perhaps related to their carriage by certain plasmids or transposable elements. It will be interesting to discover whether similar genus preferences occur in environmental isolates.

Below the level of genus preferences, we discerned in the longitudinal study a stable association of a given *mer* locus with a specific strain, regardless of the monkey in which it occurred. For example, the peripatetic ERIC types C6 and C11 always had loci 4 and 2, respectively, in monkeys L980, R830, and F79 (Fig. 3). In monkeys F78 and F79, ERIC type L1 always had

TABLE 3. Occurrence of integron components in multiresistant Hg^r isolates^a

Source	<i>mer</i> locus	No. of isolates tested	Avg no. of antibiotic resistances per isolate	No. of isolates positive for ^b :		Insert size(s) (kb)
				<i>intI</i>	Insert	
Humans	1	10	1.9	9	9	1.0, 1.9
	2	2	0.8	0	0	
	3	2	1.4	0	0	
	4	20	1.3	5	12	1.3, 2.2, 3.4
Monkeys	1	6	5.0	1	3	1.9
	2	3	0.0	0	0	
	3	9	0.1	0	0	
	4	59	2.2	9	28	1.0, 1.8, 3.3

^a The total number of isolates recovered is listed in Table 1.

^b Those positive for the *intI* gene were a subset of those positive for an insert (see text).

locus 4. ERIC type E16 occurred in both Hg^r and Hg^s forms; when it was Hg^r, it always had locus 4. Interestingly, however, for those ERIC types which were found in the PA collection and in either the EPS or ECOR collection, the Hg^r and Hg^s phenotypes were always different. For example, ERIC type E11 was Hg^r in the EPS collection but occurred only as a Hg^s ERIC type in monkey R830. Similarly, E3 was a Hg^r strain in F78 but was Hg^s in ECOR70. Thus, these very widely distributed bacterial host ERIC types may or may not carry a *mer* locus, depending on the milieu.

Finally, individual Hg^r strains are more likely also to be multiresistant to antibiotics than are Hg^s strains. This tendency to cluster resistances in a strain is seen even more strongly for antibiotic resistances. An unexpected aspect of this resistance clustering was that some *mer* loci, notably loci 1 and 4, are much more likely to occur in strains with large numbers of antibiotic resistances. That these antibiotic resistances are conjugatively transferable with Hg^r suggests that they are closely linked on some genetic element. The Tn21 family of transposons is a genetic element in which the *mer* locus is linked to an antibiotic multiresistance element, the integron. Finding an abundance of integron-related elements in strains carrying loci 1 and 4 is consistent with carrying genetic elements related to Tn21. The precise physical distances between the *mer* locus and the integron elements in these strains are under examination.

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