Phylogeny of Mercury Resistance (*mer*) Operons of Gram-Negative Bacteria Isolated from the Fecal Flora of Primates

CYNTHIA A. LIEBERT, JOY WIREMAN, TRACY SMITH, AND ANNE O. SUMMERS*

Department of Microbiology, The University of Georgia, Athens, Georgia 30602-2605

Received 11 September 1996/Accepted 19 December 1996

Nine polymorphic *mer* loci carried by 185 gram-negative fecal bacterial strains from humans and nonhuman primates are described. The loci were characterized with specific intragenic and intergenic PCR primers to amplify distinct regions covering approximately 80% of the typical gram-negative *mer* locus. These loci were grouped phylogenetically with respect to each other and with respect to seven previously sequenced *mer* operons from gram-negative bacteria (the latter designated loci 1, 2, 3, 6, 7, 8, and $\Delta 8$ by us here for the purpose of this analysis). Six of the *mer* loci recovered from primates are similar either to these previously sequenced *mer* loci or to another locus recently observed in environmental isolates (locus 4), and three are novel (loci 5, 9, and 10). We have observed *merC*, or a *merC*-like gene, or *merF* on the 5' side of *merA* in all of the loci except that of Tn501 (here designated *mer* locus 6). The *merB* gene was observed occasionally, always on the 3' side of *merA*. Unlike the initial example of a *merB*-containing *mer* locus carried by plasmid pDU1358 (locus 8), all the natural primate loci carrying *merB* also had large deletions of the central region of the operon (and were therefore designated locus $\Delta 8$). Four of the loci we describe (loci 2, 5, 9, and 10) have no region of homology to *merB* from pDU1358 and yet strains carrying them were phenylmercury resistant. Two of these loci (loci 5 and 10) also lacked *merD*, the putative secondary regulator of operon expression. Phylogenetic comparison of character states derived from PCR product data grouped those loci which have *merC* into one clade; these are locus 1 (including Tn21), locus 3, and locus 4. The *mer* loci which lack *merC* grouped into a second clade: locus 6 (including Tn501) and locus 2. Outlying groups lacked *merD* or possessed *merB*. While these *mer* operons are characterized by considerable polymorphism, our ability to discern coherent clades suggests that recombination is not entirely random and indeed may be focused on the immediate 5' and 3' proximal regions of *merA*. Our observations confirm and extend the idea that the *mer* operon is a genetic mosaic and has a predominance of insertions and/or deletions of functional genes immediately before and after the *merA* gene. *chi* sites are found in several of the sequenced operons and may be involved in the abundant reassortments we observe for *mer* genes.

In a great variety of eubacteria, the mechanism of resistance to mercurial compounds is the reduction of Hg(II) to the volatile form Hg(0) (32). This biotransformation is mediated by an inducible NADPH-dependent, flavin adenine dinucleotide-containing disulfide oxidoreductase, the mercuric reductase. The gene encoding the mercuric reductase, *merA*, together with genes coding for Hg(II) uptake (*merT* and *merP*) and, in some loci, perhaps *merC* or *merF* and regulatory functions (*merR* and *merD*) comprise the *mer* operon (see Fig. 1) (5, 32, 33). In isolates from many distinct environments, *mer* operons have been found on plasmids (9, 15, 39) and chromosomes (21, 48) and are often components of transposons (25, 33).

Since the first nucleotide sequences of the *mer* loci of transposons Tn501 and Tn21 were published, five complete, contiguous *mer* operon sequences (4, 9, 15, 24, 25), an internally deleted *mer* locus (37), and one fragmented *mer* operon sequence (21, 22) from gram-negative bacteria have been deposited in GenBank (see Table 1 and Fig. 2). We have used these sequences to design PCR primers to amplify regions of the *mer* operon. Figure 1 illustrates the arrangement of genes in the sequenced gram-negative *mer* loci. These sequences revealed that the *mer* operon is a genetic mosaic with a novel modular

arrangement (43) of essential genes and accessory genes. For the present analysis, we define as essential components of the gram-negative *mer* locus those genes which encode the regulatory protein (*merR*) (33, 35), the Hg(II) uptake system (*merT* and *merP*) (17), and the mercuric ion reductase (*merA*) (4, 8) (see Fig. 1). In addition to these essential genes, some *mer* loci also have the inner membrane protein gene, *merC* (17), whereas others have *merF*, a newly discovered gene of unknown function (20). Others have *merB*, encoding the organomercurial lyase (6, 15). Here we designate *merC*, *merF*, and *merB* as accessory genes since they are found in some but not all examples of the *mer* operon. None of the published contiguous *mer* operon sequences lack *merD*, a gene which appears to antagonize the activation function of *merR* (9), so initially we considered *merD* to be an essential gene. However, in this study, we observed contiguous *mer* loci without *merD*.

Historically, the presence of the accessory gene *merB* was first used in classification schemes of *mer* operons (27, 49). Resistance only to inorganic mercury compounds was called "narrow spectrum," and resistance to both inorganic and organic mercurial compounds was called "broad spectrum" (27, 49). The organomercurial resistance of the latter results from the action of the *merB* gene product, the organomercurial lyase. *merB* probes have been used to detect the presence of organomercurial lyase genes in environmental bacterial communities (3, 31). The first sequenced *mer* operon carrying *merB* (15) had the gene organization *merRTPABD* (see Fig. 2). A narrow-spectrum operon was located nearby on the same plas-

* Corresponding author. Mailing address: Department of Microbiology, The University of Georgia, Athens, GA 30602-2605. Phone: (706) 542-2669. Fax: (706) 542-6140. E-mail: Summers@BSCR.UGA.EDU.

TABLE 1. Sources of DNA sequence data for primer design and host strains carrying reference *mer* loci used as controls for PCR and Southern hybridizations

Designated <i>mer</i> locus type	Source of DNA sequence data for primer design				Strain and reference <i>mer</i> locus used as control for PCR and Southern hybridization		
	Common name	Source and <i>mer</i> genes sequenced	Accession no.	Reference or source	Host strain ^a	Plasmid	Source (reference)
1	Tn21	Plasmid NR1 from <i>Shigella flexneri</i> ; <i>merRTPCA</i>	K03089	4	SK1592	pDU202	T. Foster (13)
2	Tn5053	Plasmid R100 from <i>E. coli</i> ; <i>merRTPCAD</i>	J01730	33, 34	SE23	pMER419	D. Ritchie (38)
		Plasmid pMER327/419 from <i>Pseudomonas fluorescens</i> ; <i>merRTPFA</i>	X73112	20			
		Tn5053, <i>Xanthomonas</i> sp. strain W17 chromosome; <i>merRTPFAD</i>	L40585	25			
3	pKLH2	Plasmid pKLH2 from <i>Acinetobacter calcoaceticus</i> ; <i>merRTPCAD</i>	L04303	24	JM83	pKLH2.5	V. Nikiforov (24)
6	Tn501	Plasmid pVS1 from <i>Pseudomonas aeruginosa</i> ; <i>merRTPAD</i>	X03406	33, 34	GD86	None ^b	G. Dougan
7	<i>Thiobacillus</i>	Strain E-15 <i>Thiobacillus ferrooxidans</i> chromosome; <i>merR2</i> , <i>merC2</i> , <i>orf1</i> , <i>orf2</i> , <i>orf3</i> , <i>merC1</i> , <i>merR1</i>	X57326	21			
		<i>urf</i> , <i>merC</i> , <i>merA</i>	D90110	22			
8	pDU1358	Plasmid pDU1358 from <i>Serratia marcescens</i> ; <i>merRTPA</i>	M24940 ^d	35	J53	pDU1358 ^c	H. Griffin (15)
		<i>merA</i>	Z49200 ^f	GenBank	SK1592	pHG103 ^e	M. Winfrey (15)
		<i>merABD</i>	M15049 ^g	15			
Δ8	R831b (OMR)	Plasmid pCT4 from <i>E. coli</i> R831b; <i>merRTAB</i>	U77087	GenBank	J53	R831b ^h	H. Ogawa (37)
				46	SK1592	pCT4 ⁱ	C. Tolle (45)

^a All host strains are *E. coli* K-12 except SE23 which is *Pseudomonas testosteroni*.

^b Tn501 is located on the chromosome in GD86.

^c There are two *mer* loci on pDU1358: the sequenced broad-spectrum *mer* locus and an unsequenced narrow-spectrum *mer* locus (Tn21-like).

^d The end of this sequence overlaps the beginning of sequence Z49200.

^e This subclone carries only the broad-spectrum *mer* locus of pDU1358.

^f This sequence completes the gap in the previously incomplete *merA* sequence of pDU1358.

^g The beginning of this sequence overlaps the end of sequence Z49200.

^h There are two *mer* loci on R831b: the organomercurial-resistant (OMR) broad-spectrum *mer* locus and an unsequenced narrow-spectrum *mer* locus (locus 4).

ⁱ This subclone carries only OMR, the broad-spectrum *mer* locus found on a 9.4-kb *Sall*-*Xho*I fragment from R831b.

mid, pDU1358. A second example, the organomercurial resistance locus of plasmid R831b, consisted of an internally deleted *mer* operon, *merRTAB* (see Fig. 2) (45), located ca. 15 kb from a complete narrow-spectrum *mer* locus on the same plasmid (37). Recently, restriction mapping of the *mer* loci of a *Pseudomonas stutzeri* plasmid, pPB (41), has revealed still another broad-spectrum operon arrangement, *merRBTPCAD*. A narrow-spectrum *mer* locus, *merRTPAD*, was also located on plasmid pPB.

When their DNA sequences became available, it was discovered that certain narrow-spectrum resistance loci possessed an additional gene, *merC*, located between *merP* and *merA*. However, no gross phenotypic distinction could be ascribed to the presence of *merC* (17). Gilbert and Summers (14) used the presence or absence of *merC* to distinguish between Tn21-like loci (which have *merC*) and Tn501-like *mer* loci (which lack *merC*) in human fecal flora and in environmental isolates.

Moving beyond the simple *merB/merC* distinctions, Jobling et al. (23) made the first extensive phylogenetic analysis of the *mer* loci in conjugative plasmids in freshwater aquatic bacteria. Using restriction fragment length polymorphisms (RFLPs) of the *merR* to *merP* region, they observed three major types of *mer* locus as exemplified by those carried on plasmids pMER11, pMER610, and pMER419. Plasmid pMER419 has a completely novel gene, *merF*, present between *merP* and *merA* (25). The *merF* gene was originally found in the chromosome of a *Xanthomonas* species in transposon Tn5053 (20). The pMER419 and Tn5053 *mer* sequences (see Table 1) are 99.8% similar (six mismatches out of 3,056 bases) and are represented in Fig. 2 as the Tn5053 *mer* locus. Osborn et al.

(38) refined and extended the description of *mer* variation in these environmental bacteria by using PCR primers based on conserved regions in the *merR* and *merP* genes of Tn21, Tn501, and plasmid pMER419. RFLP analysis (38) of these PCR products from cultured strains was used to define 10 distinct *mer* loci (classes A to J) in gram-negative soil bacteria. Recently, Bruce et al. (10) identified 12 additional RFLP classes (K to V) recovered by PCR from total DNA extracts of soil and sediment samples.

Phylogenies based on *merR-merP* regions of these Hg-resistant (HgR) environmental isolates have been constructed, but none, as yet, have been determined for complete *mer* operons or those carried by primate microbial flora. In the study reported here, we describe nine distinct polymorphic *mer* loci carried by gram-negative fecal bacteria from humans and non-human primates. The loci were characterized with specific intragenic and intergenic PCR primers to amplify distinct regions of the typical gram-negative *mer* locus, and phylogenies based on character states were derived. Five of the six previously sequenced *mer* loci were also analyzed with the same techniques and served as standards for this analysis.

MATERIALS AND METHODS

Bacterial strains. Mercury-resistant bacterial strains from the Primate Amalgam collection (PA) ($n = 119$) (44), the Environmental Plasmid Survey collection (EPS) ($n = 59$) (14, 28), and the ECOR collection ($n = 7$) (36) were studied. These collections are described in detail by Wireman et al. (50). Fifteen strains carrying representative natural primate *mer* loci were selected for detailed description (see Tables and Figures below). The mercury-resistant strains used as standards are listed in Table 1. Two of these reference strains, J53(pDU1358) and J53(R831b), each carry two *mer* loci on a single large plasmid. From earlier

TABLE 2. Primer sequences for PCR analysis of *mer* loci

<i>mer</i> primers ^a	Direction ^b	Nucleotide sequence (5' to 3')	GenBank accession number ^c	Position
R1	F	GCG GAT TTG CCT CCA CGT TGA	K03089	458–477
R2	F	ACG GAT GGT CTC CAC ATT G	X03406	474–492
T1	R	CCA GGC AGC AGG TCG ATG CAA G	K03089	682–661
T2	R	CGA GGC AGC AAG CCG AGG CG	X03406	698–679
P1	F	GGC TAT CCG TCC AGC GTC AA	X03406	1232–1251
P2	R	TGC GGG CTA CCC ATC ATC AG	X73112	1250–1269
C1	F	CAT CGG GCT GGG CTT CTT GAG	K03089	1406–1386
C2	R	CAT CGT TCC TTA TTC GTG TGG	K03089	1750–1730
F1	F	CTC GTC GCG CTG TGT TGC TTC	X73112	1331–1351
F2	R	CAT CGG CTT GGC GTT TTC GTT	X73112	1490–1470
A0	R	GTC GCA GGT CAT GCC GGT TAT TTT	X03406	1365–1342
A1	F	ACC ATC GGC GGC ACC TGC GT	K03089	2140–2159
A2	R	GGG TGG CGC AGG ATG TGC AG	X73112	1573–1554
A5	R	ACC ATC GTC AGG TAG GGG AAC AA	K03089	3375–3357
A6	F	GCC GAC CAG TTG TTC CCC TAC CTG ACG	K03089	3346–3372
B1	F	TCG CCC CAT ATA TTT TAG AAC	M15049	382–402
B2	R	GTC GGG ACA GAT GCA AAG AAA	M15049	883–863
D1	R	CGC ACG ATA TGC ACG CTC ACC C	M15049	1186–1165
D2	F	CCA GGC GGC TAC GGC TTG TT	X03406	3138–3157
D3	R	GGT GGC CAA CTG CAC TTC CAG	X03406	3356–3336

^a Primer pairs are identified in Table 3.^b F, forward; R, reverse.^c References are listed in Table 1.

studies, single *mer* locus standards had been constructed from these strains, and we characterized these single broad-spectrum *mer* loci (locus 8 or locus Δ8; Table 1) in these subclones.

Mercury resistance. Resistances to 100 μM HgCl₂ (Hg) and 25 μM phenylmercuric acetate (Pm) were determined by replica plating (using a secondary master to avoid overinoculation) of duplicate colonies of each strain, followed by streaking for isolation of colonies on Hg or Pm Luria broth (LB) agar plates (44).

PCR primers. Oligonucleotide primers were designed by using OLIGO software (National Biosciences, Inc.) and were synthesized by the University of Georgia Molecular Genetics Instrumentation Facility, Athens, Ga., or BioServe Biotechnologies, Laurel, Md. Sequences of the primers are listed in Table 2, and the priming sites and regions of the *mer* locus which they would amplify are depicted in Fig. 1 and Table 3. The Tn21 R1-T1 primers do not amplify the *merRT* region of the Tn501 *mer* locus, and the Tn501 R2-T2 primers do not amplify the *merRT* region of the Tn21 *mer* locus. The C1-C2 primers, based on the Tn21 *mer* locus, amplify the *merC* gene of Tn21 which is absent from the four other standard loci: Tn501, Tn5053, pDU1358, and R831b. Primers F1 and F2, based on sequences from the *mer* locus on plasmid pMER419, amplify the *merF* gene, and primers B1 and B2, based on sequences from the *mer* locus on plasmid pDU1358, amplify the *merB* gene. The *merA* primers and the *merD* primer, D1, were designed from consensus regions of all known *mer* sequences and amplify all of the standard *mer* loci used in this study. The primers P1 and A0 were designed to amplify the *mer* loci of Tn21, pKLH2, Tn501, and pDU1358, and the

primers P2 and A2 were designed to amplify the *mer* locus of Tn5053. Primers D2 and D3, based on sequences from Tn501, amplify the *merD* gene of the Tn501, pDU1358, and R831b loci. Primers, PCR annealing temperatures, and product sizes are listed in Table 3.

PCR. Amplification reactions were carried out in a total of 75 μl containing 10 or 20 pmol of each primer, 0.2 mM each deoxynucleoside triphosphate, 2.5 μM MgCl₂, 2.5 or 1.25 U of *Taq* DNA polymerase (Promega, Madison, Wis.), and 1× PCR buffer (50 mM KCl, 10 mM Tris-HCl [pH 9.0], 0.1% Triton X-100). A modification of the “hot start” technique was employed with wax beads (47). For use as templates, cells were picked from plates grown overnight by touching growth with a sterile toothpick and transferred into 25 μl of nuclease-free deionized H₂O. One wax bead was added to each sample, which was then heated to 95°C for 5 min to melt the wax (and lyse the cells) and cooled to 25°C for 1 min to solidify the wax. Remaining reagents were added on top of the wax layer. Cycles were 1 cycle at 95°C for 2 min, the appropriate annealing temperature (see Table 3) for 2 min, and 72°C for 3 min; 29 cycles at 95°C for 1 min, the annealing temperature for 2 min, and 72°C for 3 min; and 1 cycle at 72°C for 5 min; products were then finally held at 15°C. For each PCR primer pair, the optimum annealing temperature calculated by OLIGO software proved effective (see Table 3). To visualize the PCR product, 5 to 10 μl of each reaction mix was electrophoresed on 1 or 2% agarose gels in 1× TBE (90 mM Tris base, 90 mM boric acid, 2 mM EDTA [pH 8.0]), stained with 0.1 mg of ethidium bromide

mer Locus and PCR Primers

FIG. 1. Locations of the *mer* PCR primers (see also Table 4). The direction of the primer is indicated by the arrowhead (i.e., forward or top strand pointing rightward and the reverse or bottom strand pointing leftward). Primer pairs are identified in Table 3.

(EtBr) per ml, and photographed. Appropriate positive and negative controls were included in every PCR run.

Restriction fragment length polymorphisms of *merA* PCR products. RFLPs were determined for all 5 standard and all 185 natural primate *mer* A1-A5 amplicands. PCR products were digested directly (separate single digests) with restriction enzymes *AluI*, *EcoRI*, and *NciI* (Boehringer Mannheim, Indianapolis, Ind.). The reactions were electrophoresed on 2 or 3% MetaPhor gels (15 by 15 cm) (FMC BioProducts, Rockland, Me.) or 2% agarose gels in 1× TBE at 100 V for 4.5 h (MetaPhor gels) or 2.5 h (agarose gels). The gels were stained with EtBr and photographed.

Mapped restriction site polymorphisms (MRSPs) of *merA* PCR products. MRSPs (40) were determined in the PCR products from the *merA* gene (A1-A5 amplicands) of one representative of each *mer* locus. In separate 10-μl end-labeling reactions, 10 pmol of primer A1 or A5 was incubated with 10 pmol of [γ -³²P]ATP (10 mCi/ml) (New England Nuclear, Beverly, Mass.), 5 U of T4 polynucleotide kinase (New England Biolabs, Beverly, Mass.), and 1× kinase buffer (70 mM Tris-HCl [pH 7.6], 10 mM MgCl₂, 5 mM dithiothreitol) at 37°C for 45 min, and the mixture was heat inactivated at 70°C for 20 min (1). One or the other of the end-labeling reaction mixtures was added to a 65-μl PCR mixture containing 50 mM KCl, 15 mM Tris-HCl (pH 9.5), 0.1% Triton X-100, 1.5 mM MgCl₂, 0.2 mM each deoxynucleoside triphosphate, 1.25 U of *Taq* DNA polymerase (Promega), and 10 pmol of the relevant unlabeled primer (final volume, 100 μl). In a separate 0.5-ml Eppendorf tube, a 25-μl suspension of cells and a wax bead were heated to 95°C for 5 min, as described above. The amplification mixture including the labeled primer was added on top of the wax layer. PCR cycles were performed as described above. End-labeled PCR products were purified using the Magic PCR Prep Kit (Promega). Partial digestion of the labeled amplicand (in a 70-μl single enzyme reaction mixture) was carried out with 1 U of enzyme in three separate single digests (*AluI*, *BsrFI*, or *NciI*) (Boehringer Mannheim) at 37°C. A 10-μl aliquot was removed before addition of the enzyme, and then 10-μl aliquots were removed after 0.5, 2.5, 5.0, 15, 30, and 60 min of incubation and added to tubes containing 1.5 μl of 0.5 M EDTA (pH 8.0) and 1.0 μl of 6× type II loading dye (42). These partial-digest aliquots were electrophoresed on 2% MetaPhor gels (15 by 15 cm) (FMC BioProducts) in 1× TBE at 100 V for 4.5 h, and the gels were stained with EtBr and photographed. Gels were air dried for 4 h with heat (GelAir Dryer, Bio-Rad, Hercules, Calif.) and exposed to X-ray film.

Isolation of total DNA and plasmid DNA. Total cellular DNA was isolated by using the IsoQuick Nucleic Acid Extraction Kit (ORCA Research, Inc., Bothell, Wash.). Plasmid DNA was extracted from 2.5 ml or 25 ml of culture with the Wizard Minipreps DNA Purification System (Promega) or the Plasmid Midi Kit (Qiagen, Inc., Chatsworth, Calif.), respectively.

Conjugation experiments. Several HgR strains representing each *mer* locus were conjugated with Hg-susceptible (HgS) and nalidixic acid (Nx)- and rifampin (Rf)-resistant recipients. Laboratory strains used as recipients were Nx- and Rf-resistant strains of *Escherichia coli* K-12, C600 (B. Bachman, *E. coli* Genetic Stock Center); *Pseudomonas aeruginosa* PAO (G. Jacoby); and *Pseudomonas putida* RKT2440 (M. Schell). All recipients were resistant to 100-μg/ml Rf and 50-μg/ml Nx. Spontaneous Rf- and Nx-resistant mutants were also derived from HgS, antibiotic-susceptible strains isolated from the primate fecal flora and were also used as recipients (*E. coli*, 690FNR; *Klebsiella oxytoca*, 538FNR; and *Enterobacter/Citrobacter* sp., 529FNR). All donors were resistant to 100 μM Hg and to either 25-μg/ml streptomycin (Sm) or 25-μg/ml chloramphenicol (Cm) (or both). Each overnight donor and recipient culture were inoculated (5 μl each) together on LB agar, incubated for 24 h at 37°C, and then streaked on the appropriate double-selective medium: 50 μM Hg and 100-μg/ml Rf, 25-μg/ml Sm and 100-μg/ml Rf, or 25 μM Pm and 100-μg/ml Rf. Resistances to 100 μM Hg, 25 μM Pm, 100-μg/ml Rf, 50-μg/ml Nx, 25-μg/ml Sm, and 25-μg/ml Cm were determined by replica plating each transconjugant, followed by single colony isolation on Hg or Pm LB agar plates. Biotypes and host strain DNA fingerprints of the transconjugants were confirmed by the API20E system from bioMérieux Vitek, Inc. (Hazelwood, Mo.) and by the method of Herrick et al. (19), respectively.

DNA hybridizations. For hybridization probes, the appropriate PCR product (see Table 3) was cut from 0.7% low-melting-point agarose gels, cleaned with Mermaid or GeneClean (BIO 101, La Jolla, Calif.), and then labeled by random priming (42) with [α -³²P]dATP (New England Nuclear). Southern hybridization by the alkaline blotting method for charged nylon membranes (Hybond-N+; Amersham, Arlington Heights, Ill.) was performed on 1 to 2 μg of total cellular DNA digested with *EcoRI* or on 10-μl samples of undigested PCR products electrophoresed on 1% agarose gels. For comparison of hybridization intensities to the Tn21 *merC*, Tn5053 *merF*, and Tn501 *merPA* probes, equivalent DNA masses were loaded on three identical gels. Hybridizations and washes were performed by standard procedures (42) at 68°C. By using a Molecular Dynamics PhosphorImager and ImageQuant software, (Molecular Dynamics, Inc., Sunnyvale, Calif.), the intensity of the test amplicand relative to the Tn21 R1-A5 amplicand when hybridized to the Tn21 *merC* probe was determined. The relative hybridization intensities compared to the Tn21 *merC* probe were grouped into three categories: hybridization intensity of 90 to 100%, of 2 to 3%, or of 0.05 to 0.3%. Also, the intensity of the test amplicand relative to the Tn501 R2-A5 amplicand when hybridized to the Tn501 *merPA* probe was determined. The relative hybridization intensities compared to the Tn501 *merPA* probe were grouped into two categories: hybridization intensity of 90 to 100% or of 3.5 to

TABLE 3. PCR *mer* primers, amplicands, and probes

Region amplified	Primer pair	Annealing temp (°C)	Amplicand size (bp)
Tn21-like <i>merR</i> , o/p, <i>merT</i>	R1-T1	60	225 ^a
Tn501-like <i>merR</i> , o/p, <i>merT</i>	R2-T2	60	225 ^a
<i>merC</i>	C1-C2	60	365 ^a
<i>merF</i>	F1-F2	59	160 ^a
<i>merA</i>	A1-A5	64	1,238
<i>merP-merA</i>			
<i>merC</i> (-like) present	P1-A0	59	572–520
<i>merF</i> present	P2-A2	59	324
<i>merC</i> and <i>merF</i> absent	P1-A0	56	134 ^a
<i>merR-merA</i>			
<i>merC</i> (-like) present	R1-A5	62	2,920
<i>merF</i> present	R2-A5	60	2,600
<i>merC</i> and <i>merF</i> absent	R2-A5	60	2,470
R831B-like	R2-A5	60	270
<i>merB</i>	B1-B2	57	502 ^a
<i>merD</i>	D2-D3	63	219 ^a
<i>merA-merD</i>			
<i>merB</i> present	A6-D1	62	928
<i>merB</i> absent	A6-D1	62	181
<i>merR-merB</i>			
PDUMER-like	R1-B2	59	3,070
R831B-like	R2-B2	59	890–690

^a The indicated amplicands of standard strains were used as hybridization probes. To verify expected fragment sizes of amplicands used as hybridization probes, RFLP were determined. PCR products were digested directly (separate single digests) with the following restriction enzymes: *TaqI-AflI* for Tn21 *merRT*; *Sau96I* for Tn21 *merC*; *BsrFI-HaeIII* for Tn5053 *merF*; and *AluI-BsrFI* for pDU1358 *merB* (Boehringer Mannheim and New England Biolabs). The reaction mixture was then electrophoresed on 2 or 3% MetaPhor gels (15 by 15 cm) or 2% agarose in 1× TBE, stained with EtBr, and photographed.

8%. The hybridization intensities determined by using the Tn5053 *merF* probe were either very intense or completely negative so relative intensities were not determined.

Phylogenetic analysis. Discrete binary character states (1 or 0) coding the presence, length, and/or hybridization intensity of the *mer* genes were used to estimate phylogenies by the Wagner parsimony method with the branch and bound algorithm PENNY (18). Consensus trees were generated using the majority rule and strict consensus tree program CONSENSE (30); both PENNY and CONSENSE are from the PHYLIP program package (version 3.53c) (12), Genetics Computer Group, Inc. (Madison, Wis.). Default options for the PENNY and CONSENSE programs were used with the exception of the maximum number of most parsimonious trees generated per run, which was set at 5,000.

RESULTS AND DISCUSSION

Characteristics used in comparison of the *mer* operons. Our working definition of the *mer* loci is based on the presence and the order of the essential genes, *merR*, *merT*, *merP*, and *merA*, and the accessory genes, *merC*, *merF*, *merB*, and *merD*, and on the comparison of the restriction sites present in the *mer* A1-A5 PCR amplicands. Variants were characterized by the length of the R1-T1, R2-T2, P1-A0, P2-A2, A6-D1, and R1(2)-B2 PCR amplicands. Amplicands from reference *mer* loci (Table 3) were used as internal probes in Southern hybridizations to confirm that observed PCR products from natural primate isolates did contain *mer* genes.

We initially detected *mer* loci by amplifying the *merRT* and the *merA* regions of the HgR strains (see subparagraphs i and ii below). We then grouped the natural primate *mer* loci and the reference *mer* loci based on the RFLPs of the A1-A5

TABLE 4. Primate bacteria *mer* loci characterized

Locus type	Relationship to sequenced <i>mer</i> loci ^a	Ritchie groups ^b	No. of loci characterized ^c
1	Tn21-like	E	20
2	Tn5053-like	B, D, H	27
3	pKLH2-like		19
4	Group F-like	F	92
5	Novel		7
6	Tn501-like	A	15
7	None found (<i>Thiobacillus</i>)		0
8	None found (pDU1358)		0
Δ8	R831b-like		7
9	Novel		2
10	Novel		5
Total			194

^a See *mer* sequence locus names in Table 1. *merA* RFLPs and *merRT* amplicand are similar to indicated standard *mer* locus.

^b *merA* RFLP and *merRT* amplicand are similar to Ritchie's strain (38).

^c Total number of loci characterized is greater than the number of strains characterized ($n = 185$) because several strains had more than one locus. Three strains each carried loci 5 and 6. Seven strains each carried locus Δ8 plus an additional *mer* locus (locus 3, 4, or an undefined HgR locus).

amplicands. The *mer* locus types (1 to 10) were assigned to distinct RFLPs of the A1-A5 amplicands (Table 4). Figure 2 illustrates the arrangements of genes of the 10 *mer* loci described in this study: the 9 different *mer* loci found in the

primate flora and the 1 broad-spectrum *mer* locus carried by pDU1358 which was not found in the primate flora. The PCR products of the reference and natural primate *mer* loci are presented in Table 5.

Our characterizations of the primate flora *mer* loci were first done in the original isolates. However, it became apparent that, like the standard strains carrying pDU1358 and R831b, some primate strains carrying loci 2, 3, 4, 5, 6, and 8 had at least one additional *mer* locus. So, we attempted conjugation or transformation to move a single locus into a plasmid-free host. This process was successful only for strains carrying locus 4. The remaining isolates which we could not resolve by transfer (ca. 19% of the primate strains studied) fell into three groups. One group carried locus 2 (which was also found as a single locus in other strains) and another locus which we have not yet defined. The second group of unresolved isolates carried locus Δ8 and another *mer* locus, either locus 3 or 4 or an undefined *mer* locus. The third group of unresolved isolates carried loci 5 and 6. We discerned elements of loci 2, Δ8, and 6 in these multiple locus strains by comparing them to the following single locus derivatives: Tn5053 in SE23, pCT4 in SK1592 and Tn501 in GD86, respectively (Table 1).

(i) *merR*, operator-promoter (o/p), and *merT*. The promoter-proximal regions of *merR* and *merT* differ sufficiently in Tn21 and Tn501 that PCR primer pairs could be chosen which amplified the o/p region from only one or the other but not both

FIG. 2. Representations of the previously sequenced, gram-negative *mer* loci carried by transposons Tn21, Tn5053, and Tn501 and plasmids pKLH2, pDU1358, and R831b (see Table 1; *Thiobacillus mer* locus [locus 7] not included) and the *mer* loci of gram-negative bacteria isolated from the fecal flora of primates. Numbers are the length (bp) of each gene. The lengths of the *merT* and *merP* genes (351 and 276 bp, respectively) are the same for all sequenced *mer* loci. Locus 7 and locus 8/pDU1358 were not found among the natural primate *mer* loci described. Loci 1, 2, and 6 gave the same amplicand sizes, Southern hybridization results, and MRSPs of the A1-A5 amplicand as the reference strains Tn21, Tn5053, and Tn501, respectively, and are each depicted here as a single locus. Locus Δ8 gave the same amplicand size and Southern hybridization result as pCT4 (R831b derivative) and is depicted here as a single locus. The different patterns of *merA* represent the 10 MRSPs observed. The vertical bar in the *merT* of locus 4 indicates the point where the tandem repeats occur. The different shadings (dark gray, medium gray, and white) of the *merC*-like genes represent the different hybridization intensities (90 to 100%, 2 to 3%, and 0.05 to 0.3%, respectively) observed when the respective P1-A0 amplicand was probed with the Tn21 *merC* amplicand. The different shadings of the *merR* genes represent the amplifiability with either the Tn21 R1-T1 primers (light) or the Tn501 R2-T2 primers (dark). The different shadings of *merD* represent whether the Tn501 D2-D3 amplicand was observed (dark) or not (light). Note that the A6-D1 amplicand provided evidence for *merD* even when the D2-D3 primers were too specific to detect it in several of the loci.

TABLE 5. PCR characteristics of wild and standard *mer* loci

Locus (loci)	Source/strain (plasmid)	<i>mer</i> amplicands with primer pairs (bp) ^a :											
		R1-T1	R2-T2	C1-C2 ^b	F1-F2	A1-A5	P1-A0	P2-A2	R1(2)-A5	B1-B2	D2-D3	A6-D1	R1(2)-B2
Standard													
Locus 1	Tn21/SK1592 (pDU202)	225	— ^c	365	—	1,230	572 ^d	—	2,920	—	—	181	—
Locus 2	Tn5053/SE23	—	225	nd ^e	160	1,230	—	324	2,600	—	—	181	—
Locus 3	JM83(pKLH2.5)	225	—	nd	nd	1,230	572 ^f	—	2,920	—	—	181	—
Locus 6	Tn501/GD86	—	225	—	—	1,230	134 ^g	—	2,470	—	219	181	—
Locus 8	SK1592(pHG103)	225	—	—	nd	1,230	134 ^h	—	2,470	502	nd	928	3,070
Loci 1 and 8	J53(pDU1358)	225	—	365	—	1,230	572, 134	—	2,470, 2,920	502	219	181, 928	3,070
Locus Δ8a	JM83(pCT4)	—	—	nd	nd	—	—	—	270	502	nd	928	890
Loci Δ8a and 4b	J53(R831b)	240	—	nd	—	1,230	560	—	270, 2,920	502	219	181, 928	890
Wild													
Locus 1	PA517H	225	—	365	—	1,230	572 ^d	—	2,920	—	—	181	—
Locus 2	PA699H	225	225	—	160	1,230	572 ^f	324	2,600	—	—	181	—
Locus 2	EPS1312A	—	225	nd	160	1,230	572 ^f , 520 ^f	324	2,600	—	—	181	—
Locus 3	PA722H	225	—	—	—	1,230	537 ^f	—	2,920	—	—	181	—
Locus 4a	EPS209A	225	—	nd	—	1,230	560	—	2,920	—	—	181	—
Locus 4b	PA533H	240	—	—	—	1,230	560	—	2,920	—	—	181	—
Locus 4c	PA742H	255	—	—	—	1,230	560 ^f	—	2,920	—	—	181	—
Locus 4d	PA665H	270	—	—	—	1,230	560	—	2,920	—	—	181	—
Locus 5	PA661H	—	225	—	—	1,230	548 ^f , 134 ^h	—	2,920	—	—	—	—
Locus 6	PA687H	—	225	—	—	1,230	134 ^g	—	2,470	—	219	181	—
Locus Δ8a and 4a	PA509H	225	—	—	—	1,230	560	—	270, 2,920	502	219	181, 928	890
Locus Δ8c	EPS1396	225	—	nd	nd	1,230	nd	nd	2,920	502	nd	181, 928	690
Locus Δ8b	EPS424C	225	—	nd	nd	1,230	nd	nd	2,920	502	nd	181, 928	720
Locus 9	EPS298B	—	225	nd	—	1,230	520 ^f	—	—	—	—	181	—
Locus 10	EPS317B	—	225 ^j	nd	—	1,230	548 ⁱ	—	—	—	—	—	—

^a Sizes >1,000 bp are approximated by comparison to amplicands from sequenced loci.

^b Primers C1-C2 only amplified the Tn21 *mer* loci and use of them was discontinued early in the study.

^c —, no amplicand produced.

^d 90 to 100% relative hybridization intensity (estimated by PhosphorImager and ImageQuant software) of a natural primate P1-A0 amplicand (large) compared to the Tn21 P1-A0 amplicand when probed with the Tn21 *merC* probe.

^e nd, not done.

^f 0.05 to 0.3% relative hybridization intensity (see footnote *d* for details).

^g 90 to 100% relative hybridization intensity (estimated by PhosphorImager and ImageQuant software) of a natural primate P1-A0 amplicand (small) compared to the Tn501 P1-A0 amplicand when probed with the Tn501 *merPA* probe.

^h 3.5 to 8% relative hybridization intensity (see footnote *g* for details).

ⁱ 2 to 3% relative hybridization intensity (see footnote *d* for details).

^j R2-T2 amplicand observed only after Southern hybridization and probing with labeled reference amplicand.

of these loci. We found that the *mer* loci amplified with the Tn501 R2-T2 primers occurred only in the *Pseudomonadaceae* (50) and those which amplified with the Tn21 R1-T1 primers occurred only in the *Enterobacteriaceae* (50). Between them, these two primer sets amplified the *merR*, *o/p*, and *merT* regions in 97% of the mercury-resistant isolates in this study. Interestingly, 54% of the R1-T1 PCR products from strains in the PA collection varied from the expected 225-bp product size of Tn21, and all of these had a locus 4 *merA* RFLP (see subparagraph ii). The size variation in these R1-T1 amplicands arose from one, two, or three tandem repeats of a 15-bp sequence preceding a GC-rich region in the *merT* gene. These tandem repeats were also observed in the EPS strains carrying *mer* locus 4. The *merA* RFLPs of the locus 4 variants (designated loci 4a, 4b, 4c, and 4d) were identical regardless of the number of tandem repeats observed in the *merT* region.

(ii) ***merA* and the RFLPs of the A1-A5 amplicands.** The primers A1 and A5 amplify 72% of the *merA* gene, which encodes the largest gene product of the operon. The 5' primer lies in the active-site region of the enzyme, and the 3' primer lies in the highly conserved unique Hg(II)-binding domain found in all MerA proteins but not any other disulfide oxidoreductases. The A1-A5 primers amplified the *merA* gene in 97% of the mercury-resistant primate isolates in this study. Single digests of the A1-A5 amplicands with *AluI* and *NciI*

(Fig. 3) revealed eight distinct RFLPs from the primate strains (Table 4).

The RFLP of the *mer* locus carried by the natural primate strains which was identical to the RFLP of the Tn21 *mer* locus was designated locus 1. The RFLP of the *mer* locus carried by the natural primate strains which was identical to the Tn501 *mer* locus was designated locus 6. The *merA* RFLP which we designated locus 2 was the same as predicted for the published sequence for Tn5053 and was only one *NciI* site different from locus pMER419. The sequences of the Tn5053 and the pMER419 loci in the region amplified by the A1-A5 primers are 99.7% similar (four mismatches out of 1,226 bases). The *merA* RFLP which we designated locus 3 was the same as the predicted pattern for the published sequence pKLH2 with the exception of one *AluI* site. The *merA* RFLPs of loci 4, 5, 9, and 10 did not match any of the sequenced reference *mer* loci. We were unable to amplify any *merA* PCR products to characterize the *mer* locus in only 5 of the 185 HgR strains we examined.

(iii) **Presence or absence of *merC* or *merF*.** Primers anchored in the 3' region of *merP* and the 5' region of *merA* generated a PCR product which indicated the presence of the *merC* gene, the *merF* gene, or no additional gene on the 5' side of *merA* (Fig. 2). Three *merPA* PCR product sizes were observed (Fig. 4A): large (572 to 520 bp), intermediate (324 bp), and small (134 bp). The presence of *merC* in the large P1-A0 amplicands

FIG. 3. Distinct patterns of the *Nci*I fragments of the *mer* A1-A5 PCR amplicands. Lane 1, size marker (SM) (BioMarker Low; Bioventures Inc., Murfreesboro, Tenn.) and uncut Tn21/pDU202 *mer* A1-A5 amplicand; lane 2, locus 1 (Tn21/pDU202); lane 3, locus 1 (517H); lane 4, locus 2 (699H); lane 5, locus 3 (722H); lane 6, locus 4 (742H); lane 7, locus 5 (661H); lane 8, locus 6 (687H); lane 9, locus 6 (Tn501/GD86); lane 10, locus 8 (pHG103); lane 11, locus 9 (298B); lane 12, locus 10 (317B). See Materials and Methods for a description of the conditions used. Strains are those listed in Table 5; the prefixes PA and EPS have been removed here for brevity.

was confirmed by Southern hybridization with a DNA probe specific for the *merC* gene of Tn21. We observed various intensities of hybridization of the *merC* probe to the large P1-A0 PCR product in loci 1, 3, 4, 5, 9, and 10 (Fig. 4B). The Tn21 locus (lane 1) and Tn21-like/locus 1 (lane 3) (RFLP identical to the Tn21 *merA* amplicand) gave the most intense hybridization signals compared to others (Table 5). Using the P1-A0 primers, we observed an additional *mer* locus (or fragment of a locus) present in strains carrying locus 2 (Fig. 4B, lanes 5, 7, and 9) but were unable to define it further. All the P1-A0 amplicands which hybridized to the Tn21 *merC* probe did not hybridize to the Tn501 *merPA* probe.

The presence of *merF* in the 324-bp intermediate-length P2-A2 amplicands was confirmed by Southern hybridization with a DNA probe specific to the *merF* gene of the Tn5053 locus. The *merF* gene was only detected in strains carrying the locus 2 *merA* RFLP (Fig. 4C, lanes 29, 31, 33).

The small, 134-bp, P1-A0 amplicands (Fig. 4A) occurred in strains with the *merA* RFLP patterns designated pDU1358/locus 8 (lane 23), locus 5 (lane 24), Tn501/locus 6 (lane 26), and Tn501-like/locus 6 (lane 27). Strains carrying locus 5 had both large and small P1-A0 amplicands, a large amplicand (548

bp) with a *merC*-like size gene which hybridized to the Tn21 *merC* probe, and a small amplicand (134 bp) which did not hybridize to the Tn21 *merC* probe, the Tn5053 *merF* probe, or the Tn501 *merPA* probe. All of these 134-bp, P1-A0 amplicands were also negative for both *merC* and *merF* hybridization probes (Fig. 4B and C, lanes 23 to 27). Only the Tn501/locus 6 and Tn501-like/locus 6 P1-A0 amplicands were positive (hy-

FIG. 4. P1(2)-A0(2) PCR products separated in a 1.5% agarose gel and stained with EtBr (A) and Southern hybridizations with the Tn21 *merC* probe (B) (picture is a composite of X-ray films exposed for different times: lanes 1 and 3, 30 min; lanes 5 to 21, 36 h; and lanes 23 to 33, 72 h), the Tn5053 *merF* probe (C), and the Tn501 *merPA* probe (D). P1-A1 amplicands are in lanes 1 to 27. P2-A2 amplicands are in lanes 29 to 33. Lanes 1, locus 1 (Tn21/pDU202); lanes 2, size marker (SM) (BioMarker Low); lanes 3, locus 1 (517H); lanes 5, undefined (U) locus (SB12); lanes 7, U locus (699H); lanes 9, U locus (1312A); lanes 11, locus 3 (pKLH2.5); lanes 13, locus 4 (742H); lanes 15, locus 10 (317B); lanes 16, no *mer* locus (SK1592); lanes 17, locus 3 (722H); lanes 19, locus 3 (2703); lanes 21, locus 9 (298B); lanes 23, locus 8 (pHG103); lanes 24, locus 5 (661H); lanes 26, locus 6 (Tn501/GD86); lanes 27, locus 6 (687H); lanes 28, SM; lanes 29, locus 2 (SB12); lanes 31, locus 2 (699H); lanes 33, locus 2 (1312A). (Lanes not specifically identified were empty.) Strain names are as designated in the legend to Fig. 3.

FIG. 5. (A) A6-D1 PCR products separated in a 1.0% agarose gel and stained with EtBr. (B) Southern hybridization with the pHG103-derived *merB* probe. Lanes 1, locus 1 (Tn21/pDU202); lanes 2, locus 1 (517H); lanes 3, locus 3 (722H); lanes 4, locus 10 (317B); lanes 5, locus 9 (298B); lanes 6, locus 4 (742H); lanes 7, locus Δ8 (509H); lanes 8, locus Δ8 (R831b/pCT4); lanes 9, locus 5 (661H); lanes 10, locus 6 (687H); lanes 11, locus 6 (Tn501/GD86); lanes 12, locus 8 (pDU1358); lanes 13, locus 2 (699H); lanes 14, locus 2 (1312A); lanes 15, size marker (SM) (BioMarker Low and lambda *Hind*III). Strain names are as designated in the legend to Fig. 3.

bridization intensities of 90 to 100%) when hybridized with the Tn501 *merPA* probe (Fig. 4D, lanes 26 and 27).

(iv) **Presence or absence of *merB* and *merD*.** We selected 33 of the 67 Pm-resistant (PmR) strains to examine for the presence of *merB*. Strains were chosen to include representatives of all 12 PmR biotypes recovered (50). The presence of *merB* was assessed by Southern hybridization, with the pHG103-derived *merB* amplicand as probe, of *Eco*RI-digested total DNA. Seven of 33 (18%) PmR strains produced a hybridization signal indicating the presence of *merB*. From these same seven strains we observed a 502-bp amplicand with the B1 and B2 primers. PCR primers anchored in *merA* and in *merD* produce a 928-bp amplicand when *merB* is present (Fig. 5A, lanes 7, 8, and 12) or a 181-bp amplicand when *merB* is absent (Fig. 5A, lanes 1, 2, 3, 5, 6, 10, 11, 13, and 14). Four of the seven PmR

strains whose total DNA hybridized to the *merB* probe produced such a 928-bp A6-D1 amplicand, and each hybridized to the *merB* probe (e.g., Fig. 5B, lanes 7 and 8).

Unlike the standard locus carried by pDU1358, which we call locus 8, the *merB* gene in all of the natural primate strains we examined occurred only in loci with large internal deletions, probably extending from *merT* through *merA*. This was discerned by using the primers R1(2) and B2, with which we can detect a 3,070-bp amplicand in locus 8 (data not shown). With these same primers, we found considerably smaller amplicand sizes in the several *merB*-containing strains we examined. Three amplicand sizes were observed, 890, 720, and 690 bp (designated loci Δ8a, Δ8b, and Δ8c, respectively), suggesting different extents of deletion between *merR* and *merB*. Hybridizing these amplicands with the *merB* probe confirmed the presence of the *merB* gene. All of the *merB*-positive loci we examined were thus more similar in this respect to the internally deleted locus of plasmid R831b (46), which we call locus Δ8, than to the complete locus of plasmid pDU1358 (15).

Five of the strains which carried locus Δ8 also carried another *mer* locus which we could describe; four of them carried the *mer* locus 4a and one of them carried locus 3. We were unable to amplify sufficient *mer*-specific PCR products to characterize the additional *mer* locus(i) of one of the strains carrying locus Δ8. We observed locus Δ8 in three biotypes: *E. coli*, *Citrobacter freundii*, and *K. oxytoca* (50).

We first suspected that locus 5 and locus 10 did not have *merD* when no A6-D1 PCR product was detected (Fig. 5A, lanes 4 and 9). Southern hybridization with the Tn501 *merD* amplicand as probe confirmed that all the natural primate and standard strains possessed *merD* except those carrying locus 5 or locus 10 (data not shown).

(v) **Genetic linkage of the *mer* loci.** The primer pair R1-A5 or R2-A5 was used to amplify approximately 80% of the *mer* locus to establish the physical contiguity of the individual genes detected above. The R1(2)-A5 amplicands were probed with Tn21 *merC* and pMER419 *merF* amplicands to confirm whether the 2,920-bp product contained *merC*, the 2,600-bp product contained *merF*, and the 2,470-bp product lacked both *merC* and *merF*. As noted above, a very small R1(2)-A5 product (e.g., 270 bp) was taken as evidence of the internally deleted *mer* operon (locus Δ8); such abbreviated operons were found in all the strains we examined which were PmR and carried the *merB* gene.

(vi) **Comparison of the primate *mer* loci with those from environmental samples.** Use of the above strategies on strains representing Ritchie classes A to J (38) showed that class A (SB8) corresponds to Tn501/locus 6 and class E (SE20) corresponds to Tn21/locus 1 (Table 4). Classes F (SE31) and G (SO5) resembled loci 4d and 4a, respectively. Classes B (SE23), D (SE12), and H (SB12) were similar to strains which carry Tn5053/locus 2, i.e., they were all *merF* positive, they produced both a 2,600-bp R2-A5 amplicand and a 324-bp P2-A2 amplicand, and their *Alu*I and *Nci*I *merA* RFLPs were like those of the sequenced Tn5053 locus. Ritchie classes C (SE6), I (T2:7), and J (T2:19) had *Alu*I and *Nci*I *merA* RFLPs which were not observed in the primate *mer* loci characterized. Conversely, the *merA* RFLPs of loci 3, 5, 9, and 10 were not observed in any of the Ritchie A to J classes.

***mer* phylogeny.** We performed two separate phylogenetic analyses: one was based on the binary character states of the PCR amplicands described above and another was based on the binary character states of the MRSPs of the *merA* amplicands. Wagner parsimony (11, 26) is a qualitative method which finds all the most parsimonious trees (shortest total branch lengths) by using discrete character states. Branch

lengths reflect the number of evolutionary changes along each ancestral-descendant pathway. Unlike quantitative distance methods, Wagner parsimony infers branches of varying unit lengths based on the number of differences (steps) between operational taxonomic units. We selected this algorithm since a most parsimonious tree is one that requires the smallest number of evolutionary changes to explain the observed differences among operational taxonomic units (2).

(i) **Character states based on the PCR amplicands.** For each standard or natural primate *mer* locus, the *merRT* amplicand type (Tn21-like or Tn501-like) and its length were coded as discrete character states. The P1-A0 and P2-A2 amplicand sizes and hybridization intensities (Table 5) were coded as discrete character states. For each *mer* locus, the presence of *merD* and the A6-D1 and R1(2)-B2 amplicand sizes were also coded as discrete character states. The distantly related *Thiobacillus* sequence was used as the outroot *mer* locus for the character states derived from the PCR amplicand data.

An estimate of a maximum parsimony tree based on the data matrix, *mer* locus types by PCR amplicand-encoded characters, is presented in Fig. 6A. Phylogenetic comparison of the character states derived from PCR amplicand data grouped *mer* locus 1 (including Tn21), locus 3, and locus 4 (i.e., loci which have *merC* or a *merC*-like gene) into one clade and grouped *mer* locus 6 (including Tn501) and locus 2 (i.e., both loci which lack *merC*) into another clade. Although locus 9 contained a *merC*-like region, it was placed in the "no *merC*" clade in 30% of the most parsimonious trees generated for this data set. Loci grouped with the "*merC*" clade all produce the R1-T1 amplicand and are found in enteric host strains (50). Loci grouped with the no *merC* clade all produce the R2-T2 amplicand and are found in *Pseudomonas* host strains (50). The outliers from these two clades were those which lacked *merD* (loci 5 and 10) or those which possessed *merB* (pDU1358, R831b, and the $\Delta 8$ loci). Thus, on the basis of presence or absence of genes, the two most thoroughly studied examples of the *mer* locus, Tn21 and Tn501, represent two major clades found in both environmental isolates and those from the normal flora.

(ii) **Character states derived from MRSPs of the *merA* amplicand.** Partial digestion of end-labeled A1-A5 amplicands allowed the positions of all 36 *AluI*, *BsrFI*, *EcoRI*, and *NciI* sites to be determined. These restriction site positions were consistent with the fragment lengths we observed in each locus. The *merA* MRSPs of the eight loci found in the primate flora and of the four additional reference loci, pKLH2, pMER419, pDU1358, and *Thiobacillus*, were coded as discrete character states for phylogenetic analysis (Table 6). The *Thiobacillus* sequence was used as the outroot *mer* locus for the character states derived from the MRSPs of the *merA* A1-A5 amplicand data. The R831b/locus $\Delta 8$ with its internal deletion of *merA* was not included.

Interestingly, separation of Tn21-like and Tn501-like *mer* loci of the PCR amplicand character state data was not observed in the phylogenetic comparison of the *merA* MRSP character data (Fig. 6B). In 80% of the most parsimonious trees generated for the MRSP data set, Tn21 and Tn501 *mer* loci were grouped together. This clade also contained loci 2, 5, 9, and 10 and the Tn5053 locus. The presence and/or absence of the *merC*, *merF*, and *merD* genes in these loci varied considerably. Two distinct outliers from this clade were locus 4 and the pDU1358 locus. The pKLH2 locus and locus 3 grouped together but were outside all other *mer* loci with the exception of the *Thiobacillus* locus.

In summary, of the nine *mer* loci identified in bacteria cultivable from the primate flora, six are similar to previously sequenced loci or to recent environmental isolates and three

FIG. 6. (A) The *mer* phylogeny based on the presence or absence of genes. Nine most parsimonious trees were found by the Wagner parsimony method using the branch and bound algorithm PENNY/PHYLP, version 3.53c. A consensus tree was produced by the majority rule and strict consensus tree program CONSENSE/PHYLP, version 3.53c. The numbers at the forks of the tree indicate the percentage of trees in which the group consisting of the loci which are to the right of that fork occurred. (B) The *mer* phylogeny based on MRSPs of *merA*. Thirty most parsimonious trees were found by the Wagner parsimony method using the branch and bound algorithm PENNY/PHYLP version 3.53c. A consensus tree was produced by the majority rule and strict consensus tree program CONSENSE/PHYLP, version 3.53c. The numbers at the forks of the tree indicate the percentage of trees in which the group consisting of the loci which are to the right of that fork occurred.

are novel loci. It is not uncommon for there to be more than one Hg resistance locus per strain. This was notably the case for the five organomercury-resistant strains examined in this study which carried both an internally deleted locus consisting of *merR*, *merB*, and *merD* with various amounts of the intervening genes and a full-length *mer* operon lacking *merB* and having a *merC*-like region.

The fact that the primers based on the *merA*, *merR*, or *merT* gene each amplified 97% of the cultivable HgR strains suggests that at least in the regions represented by these primers there is considerable conservation in these essential genes of the *mer* operon. However, regions on either side of the *merA* gene are rife with variation. On the 5' side, between *merP* and *merA*, we found either no additional genes at all, genes closely related to *merF*, or genes of the same approximate size as *merC* but with widely differing homologies to it. It remains to be determined how these observed polymorphisms affect any aspect of Hg

TABLE 6. Discrete character states of the *merA* region of the standard and natural primate *mer* loci

Locus	A1-A5 MRSP ^a																																				
	N1	N2	B1	A1	A2	B2	N3	B3	A3	B4	N4	N5	A4	N6	A5	N7	N8	A6	E1	B5	N9	A7	A8	N10	B6	N11	A9	B7	B8	B9	N12	B10	A10	N13	B11	A11	
Tn21	1	0	0	0	0	0	0	0	0	1	1	1	0	0	0	0	0	1	0	1	1	1	1	0	0	1	0	1	1	1	0	0	0	1	0	0	
pMER419	1	0	0	0	0	0	0	0	0	1	0	1	0	1	0	1	1	0	0	1	0	1	0	1	0	0	1	1	1	0	0	1	1	0	0		
Locus 2	1	0	0	0	0	0	0	0	0	1	0	1	0	1	0	1	1	0	0	1	0	1	0	0	0	0	1	1	1	0	0	1	1	0	0		
pKLH2	0	0	0	0	0	0	1	0	0	0	1	1	0	1	1	0	1	0	1	1	1	0	0	0	0	0	0	1	0	1	0	0	1	1	0	0	
Locus 3	0	0	0	0	0	0	1	0	1	0	1	1	0	1	1	0	1	0	1	1	1	0	0	0	0	0	0	1	0	1	0	0	1	1	0	0	
Locus 4	1	0	0	0	0	0	0	0	0	0	1	1	1	1	0	0	1	0	0	1	1	1	0	0	0	0	1	1	0	1	0	0	1	1	0	0	
Locus 5	0	1	0	1	0	1	0	0	0	1	0	0	1	1	0	0	1	0	0	0	0	1	0	0	0	0	0	1	0	1	1	0	1	0	1	0	0
Tn501	1	0	0	0	1	0	0	0	0	0	1	1	1	1	0	0	1	0	0	1	0	1	0	0	0	0	1	1	1	1	0	0	0	0	0	0	
Locus 9	1	0	1	0	0	0	1	1	0	0	0	1	1	0	0	0	1	0	0	1	1	1	0	0	1	0	1	1	1	1	0	0	1	0	1	1	
Locus 10	1	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0	1	0	0	0	0	1	0	0	1	0	1	0	1	1	1	0	1	0	0	1	
pDU1358	1	0	0	0	0	0	0	0	0	0	0	1	0	1	1	0	0	0	0	1	1	0	0	0	0	0	1	1	0	1	0	0	1	1	0	0	
Thiobacillus	1	0	0	0	0	0	1	0	0	0	0	1	0	1	0	1	1	0	1	0	1	0	0	0	0	0	0	1	0	0	0	0	1	1	0	1	

^a *AluI* (A), *BsrFI* (B), *EcoRI* (E), and *NciI* (N) MRSPs of *mer* A1-A5 amplicand. N1 is at position 2221 and N13 is at position 3277 of the GenBank sequence of *merA* in Tn21 (K03089). Presence or absence of the enzyme restriction sites of the *merA* amplicand indicated by one or zero, respectively.

resistance. On the 3' side of *merA*, the *merB* gene can be inserted. The loci with a *merB* gene closely homologous to that of pDU1358 represented only 18% (6 of 33) of the PmR strains examined. Thus, either the organomercurial lyase gene diverges considerably or there is some other mechanism for Pm resistance.

As another example of divergence on the 3' side of *merA*, we have found *mer* loci lacking *merD*; one locus (locus 5) occurs in *Pseudomonas* and the other (locus 10) occurs in both *Pseudomonas* and *Enterobacter* (50). Thus, for the first time, we have found, in gram-negative bacteria, contiguous *mer* loci without *merD*.

The view of the *mer* operon which emerges is of a locus with relatively stable genes such as *merR*, *merT*, and *merA* interspersed with genes of known function (*merB*) or partially defined functions (*merC*, *merF*, and *merD*) which are highly variable. Whether this model of the *mer* locus as a dynamic mosaic of gene conversions is generalizable beyond these primate isolates will hopefully be tested by the work of others on environmental isolates.

The mechanistic bases of these unusual "plug and play" recombination events remain to be determined. The occasional occurrence of more than one Hg resistance locus per strain (and even per plasmid, e.g., pDU1358 and R831b) suggests there is ample opportunity for either homologous or site-specific recombination systems acting near *merA* to provide the reassortment of genes noted here. Certainly homologous recombination within the highly conserved *merA* gene will allow reassortment of *merC* or *merF* with *merB* or *merD*, and many such arrays appear among the nine loci we have observed.

A hot spot for recombination in or near *merA* could give rise to the discordant phylogenies of the *merA* gene itself and of the operons as a whole. It is noteworthy that a sequence identical to the recombination-stimulating *chi* site (5'-GCTGGTGG-3') (29) lies in the *merT* gene of the published sequences of the Tn501 and Tn5053 *mer* loci. A single base pair variant of the *chi* sequence (5'-ACTGGTGG-3') also occurs in the *merC* gene of the Tn21 *mer* locus and in the *merP* gene of the Tn501 *mer* locus. On the basis of our findings here, we hypothesize that the *mer* operon of gram-negative bacteria is a modular, mosaic structure like the bacteriophage lambda immunity region (16) or like the mosaic structure of conjugational plasmids recently described by Boyd et al. (7) which experience site- or region-specific recombination events some of which may involve *chi*-mediated homologous recombination. We are presently examining this hypothesis further.

ACKNOWLEDGMENTS

We gratefully acknowledge the cheerful and competent assistance of Lysha Cook; provision of strains by Donald Ritchie and Mike Winfrey; comments on the manuscript by Ruth Hall, Jim Herrick, Barbara Murray, Rosemary Redfield, Mark Wise, and two anonymous reviewers.

This project was partially supported by grants from NIH (DE 10784) and from the International Academy of Oral Medicine and Toxicology to A.O.S.

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