

Selection for Mercurial Resistance in Hospital Settings

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The frequency of resistance to Hg²⁺ in 1980 to 1981 collections from Barnes Hospital, St. Louis, Mo., was only 2% for *Staphylococcus aureus* and 9% for *Escherichia coli*. The frequency of Hg²⁺ resistance in *E. coli* isolates from Jikei University Hospital, Tokyo, Japan, was 57% during 1972 to 1977 and decreased to 29% in 1979 to 1982; for *S. aureus* the frequency of Hg²⁺ resistance dropped from 36% in 1972 to 1977 to 10% in 1979 to 1982. Frequencies of resistances to cadmium (*S. aureus*) and arsenic (*S. aureus* and *E. coli*) remained approximately constant during this time. The decrease in frequency of mercurial resistance is attributed to the termination of the use of organomercurials (largely phenylmercury and thimerosal) in hospital liquid detergents and disinfectants. It is proposed that selection for mercurial resistance occurred within the hospital setting when there was widespread use of mercurials. The resistance patterns and phage types for each of four new mercurial-resistant *S. aureus* isolates from St. Louis were distinct, indicating that no single type of "hospital staph" predominates. Furthermore, resistance to thimerosal, merbromin, and methylmercury and the ability to volatilize ¹⁴C from [¹⁴C]methylmercury were found with the new isolates and never with previously known mercurial resistance plasmids in *S. aureus*.

Very high frequencies of heavy metal resistances have been found in a wide range of gram-negative and gram-positive bacteria (13-16, 18, 21, 22). These frequencies ranged from 25 to >90% of fresh clinical isolates. Often, the resistances could be transferred by bacterial conjugation (14, 21, 22) and therefore were probably governed by genes on plasmids. Approximately 25% of plasmids that had already been transferred by conjugation into *Escherichia coli* K-12 from a wide range of gram-negative organisms and chosen as having transmissible antibiotic resistances also conferred resistances to Hg²⁺ and to a range of organomercurials (18). Mercury resistance is due to an enzymatic detoxification process resulting in the reduction of Hg²⁺ to volatile Hg⁰ (6, 17, 23, 24).

The classes of plasmids conferring resistances to Hg²⁺ and to organomercurials including phenylmercury, methylmercury, merbromin (Mercurochrome), and thimerosal (Merthiolate) (which are of clinical and industrial interest; structures given in reference 28) have been summarized (1, 28, 29). Quite separate systems for cadmium resistance in *Staphylococcus aureus* (16, 27, 30) and arsenic resistance in *S. aureus* and in *E. coli* (16, 20) have also been studied,

and we have a reasonable understanding of the mechanisms of resistance to Cd²⁺ (27, 30) and AsO₄³⁻ as well (19, 20).

The nature of the agents selecting for high-frequency occurrence of heavy metal resistance plasmids in hospitals is not clear. Several alternatives have been suggested, including (i) selection by mercurial use in hospitals (8); (ii) selection outside of the hospital by industrial or urban pollution or both (9, 22); (iii) heavy metal resistance genes going for a "free ride" on antibiotic resistance plasmids associated with specific types of *S. aureus* involved in human infections (26, 31); and (iv) selection from "natural sources" such as the known high mercury level in fish (3), high cadmium in cigarettes and oysters (5), and high arsenic in shrimp (2). Witte et al. (31) reported that high frequencies of Hg²⁺ resistance in *S. aureus* were specific for *S. aureus* from human hospital collections and that *S. aureus* from human outpatients, from healthy humans, or from a variety of animal sources in the German Democratic Republic did not have significant frequencies of Hg²⁺ resistance. The distinction between human *S. aureus* (high frequency of Hg²⁺ resistance) versus animal *S. aureus* (low frequency of Hg²⁺ resistance) was also found in other countries (11).

As we will show in this paper, the frequency of Hg²⁺- and organomercurial-resistant bacteria is very low at Barnes Hospital in St. Louis, Mo.,

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TABLE 1. Resistance pattern of *S. aureus* isolated in 1980 to 1981^a

Resistance	% Resistant strains		
	Blood infections	Random infections	Total
Hg ²⁺	3	1	2
Cd ²⁺	22	20	21
AsO ₄ ³⁻	50	47	49
Penicillin G	77	86	82

^a From the Clinical Bacteriology Laboratory, Barnes Hospital, St. Louis, Mo. Frozen cultures of *S. aureus* isolated from 99 human blood infections in 1980 and 118 *S. aureus* isolates from random clinical laboratory plates in 1981 were tested.

and decreased at Jikei Medical School Hospital, Tokyo, Japan, since the discontinuation of hospital mercurial use. We therefore attribute the high frequencies found earlier and elsewhere to widespread usage of mercurials.

MATERIALS AND METHODS

Previously established methods of screening for heavy metal resistances by inhibition zones surrounding paper disks on nutrient agar in St. Louis (1, 4, 28) or by the agar dilution method with incorporation into nutrient agar in Tokyo (13–15) were used. Both methods gave consistent results with clear-cut susceptibility or resistance to Hg²⁺ and no intermediate responses (e.g., reference 15). Standard laboratory *S. aureus* strains RN1 (a variant of strain 8325), RN4 (RN1 with plasmid pI524), RN23 (RN1 with plasmid pI258), U71 (with an unnamed mercuric resistance plasmid), and U71-22 (a cured mercury-susceptible variant of U71) were used as controls. These strains were described previously (16, 20, 28). *E. coli* K-12 derivative J53 without a plasmid and with plasmids R222 and R828 (17, 18) and *Pseudomonas aeruginosa* strain PAO9501 without a plasmid and with plasmid pVS1 (1) were from our laboratory collection.

Radioactive volatilization experiments (4, 17, 28) and nonradioactive mercury volatilization measured

by atomic absorption spectroscopy (1) were carried out as previously described.

RESULTS

Frequencies of heavy metal resistances. Table 1 shows the results from 99 *S. aureus* strains isolated from blood infections in 1980 and from 118 random clinical *S. aureus* isolates in early 1981 from the Washington University teaching hospital in St. Louis. There was no significant difference between the two groups; thus the data were pooled. Although frequencies of resistance to arsenate and cadmium were of the same order as in earlier hospital collections, the frequency of mercury resistance was only 2%, lower than previously reported. With 1981 hospital isolates of *E. coli* from Barnes Hospital in St. Louis, the frequency of mercuric resistance was low at only 9%, whereas arsenate resistance occurred at 24% and carbenicillin resistance occurred at 21% (126 strains tested).

A comparison of heavy metal resistance frequencies at Jikei University Hospital, Tokyo, from 1972 to 1977 with 1979 to 1982 frequencies (Table 2) shows a decrease of more than two-thirds in the frequency of mercuric resistance in *S. aureus*, with no change in the Cd²⁺ and AsO₄³⁻ resistance frequencies. There was a smaller decrease in the frequency of Hg²⁺-resistant *E. coli*. There is a problem with the frequencies of Cd²⁺ resistance in *E. coli*. In St. Louis, none of 47 tested clinical *E. coli* isolates was more resistant to Cd²⁺ than standard laboratory *E. coli* K-12 strains. In Tokyo, such *E. coli* isolates were listed as Cd²⁺ resistant since they were approximately as resistant to Cd²⁺ as plasmid-containing *S. aureus* isolates (see references 13 to 15 for actual minimal inhibitory concentrations by the agar dilution method; analogous frequency distributions of inhibition zone diameters have not been published). The problem is an arbitrary one of whether to com-

TABLE 2. Heavy metal resistance frequencies in Tokyo^a

Resistance	Yr	Frequency (%)			
		<i>S. aureus</i>	<i>E. coli</i>	<i>P. aeruginosa</i>	<i>K. pneumoniae</i>
Hg ²⁺	1972–1977	36	57	75	66
	1979–1982	10	29	69	32
Cd ²⁺	1972–1977	50	(93) ^b	(97)	98
	1979–1982	49	(90)	(97)	94
AsO ₄ ³⁻	1972–1977	49	61	99	84
	1979–1982	51	52	97	72

^a Random isolates from the Clinical Bacteriology Laboratory, Jikei University Medical School Hospital, Tokyo, Japan. Numbers tested were as follows: *S. aureus*, 515 in 1972 to 1977 and 392 in 1979 to 1982; *E. coli*, 564 and 756, respectively; *P. aeruginosa*, 787 and 573, respectively; and *K. pneumoniae*, 331 and 412, respectively.

^b Resistant to cadmium relative to the remaining 3 to 10%. The resistance levels of these strains (in parentheses) to Cd²⁺ did not exceed that of the standard *E. coli* K-12 or *P. aeruginosa* PAO strains.

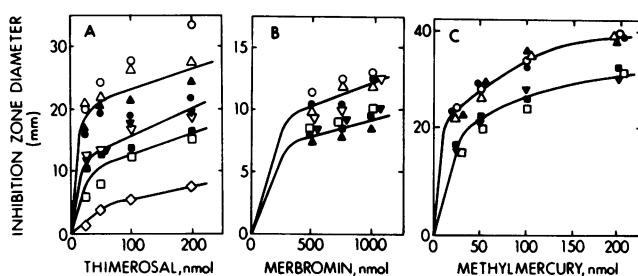


FIG. 1. Thimerosal (A), merbromin (B), and methylmercury (C) susceptibility. From 25 to 1,000 nmol of thimerosal, merbromin, or methylmercuric chloride was placed on disks on the surface of nutrient agar plates spread with 0.05 ml of overnight nutrient broth cultures. Symbols: (A) *S. aureus* strains RN1 (○), RN4 (●), U71-22 (△), U71 (▲), 1123 (□), and 1255 (■) and *E. coli* strains J53 (▽), J53(R222) (▼), and J53(R828) (◇). (B) Symbols as in (A) except for *S. aureus* strains 1585 (▽) and 3794 (▼). (C) Symbols as in (B) except for *S. aureus* strain RN23 (△). After 16 h of incubation at 37°C, the diameters of the inhibition zones surrounding the disks (minus the 6.5-mm diameter of the paper disks) were measured.

pare resistance levels within sets of clinical isolates or to compare them with standard laboratory strains.

The decrease in frequency of Hg^{2+} resistance found in *E. coli* and *S. aureus* was also found with *Klebsiella pneumoniae* (Table 2), but not significantly with *P. aeruginosa*, which has consistently shown the highest frequency of mercury resistance. We do not know whether these frequency differences from species to species are a reflection of different environmental niches or of exposure to mercurials within hospitals.

Thimerosal-resistant *S. aureus*. When we previously (28) examined resistance to organomercurials with Hg^{2+} -resistant *S. aureus* strains, all eight tested Hg^{2+} -resistant strains were susceptible to thimerosal, methylmercury, and merbromin, but were resistant to phenylmercury, fluorescein mercuric acetate, and *p*-hydroxymercuribenzoate. This pattern distinguishes the Hg^{2+} -resistant *S. aureus* from Hg^{2+} -resistant *E. coli* and *P. aeruginosa*, which were invariably resistant to merbromin and showed coupled resistance to phenylmercury and thimerosal or to neither (29). Of the new Hg^{2+} -resistant *S. aureus* isolates in St. Louis, three (strains 1123, 1255, and 3794) showed slightly more resistance to thimerosal, methylmercury, and merbromin (Fig. 1; unpublished data) than the standard

laboratory strain RN1 with plasmid pI524 (strain RN4) (Fig. 1). With careful comparisons on petri dishes, a similar significant difference in resistance level to thimerosal and merbromin was found between the well-studied laboratory *S. aureus* strain U71 (see reference 28 for earlier references), which has a mercurial resistance plasmid, and the cured derivative U71-22, isolated in St. Louis. Although the plasmid-determined resistance to thimerosal in *S. aureus* seen in Fig. 1A was reproducible, it was less than that which we previously reported in gram-negative bacteria (1, 18, 29). Figure 1A also contains results with a plasmidless *E. coli* strain J53 and the same strain with plasmids that do (R828) or do not (R222) confer thimerosal resistance. The difference between strains U71 and U71-22 was more apparent with merbromin (Fig. 1B) than with thimerosal (Fig. 1A). All 4 of the new Hg^{2+} -resistant *S. aureus* strains (Table 1) were resistant to phenylmercuric acetate (data not shown), and none of the 11 new *E. coli* isolates was phenylmercuric resistant. To date, all mercuric-resistant *S. aureus* isolates have shown phenylmercury resistance (28, 29), although very few mercuric-resistant *E. coli* isolates are phenylmercuric resistant (18, 29). Three of the four new *S. aureus* isolates showed a small resistance to methylmercury (Fig. 1C). Table 3 contains a

TABLE 3. Resistance patterns of new mercurial-resistant *S. aureus* isolates

Strain	Resistances ^a	Phage type ^b	Source
1123	Hg ^r Pma ^r Thi ^r As ^r Cd ^s Pb ^r Pen ^r	53/77/84/85	Blood infection
1255	Hg ^r Pma ^r Thi ^r As ^s Cd ^r Pb ^r Pen ^s	Not typable	Blood infection
1585	Hg ^r Pma ^r Thi ^s As ^r Cd ^r Pb ^r Pen ^r	29/6/42E/47/54/85/81/80/53 ±	Blood infection
3794	Hg ^r Pma ^r Thi ^r As ⁱ Cd ⁱ Pb ^r Pen ^r	6/47/53 ±/75	Random infection

^a Resistant (r), susceptible (s), or intermediate (i) response to mercuric chloride (Hg), phenylmercuric acetate (Pma), thimerosal (Thi), arsenate (As), cadmium (Cd), lead (Pb), or penicillin G (Pen).

^b Phage typing with standard *S. aureus* phage by R. W. Lacey (personal communication).

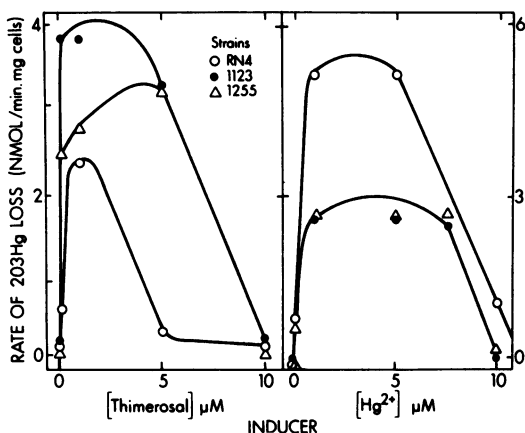


FIG. 2. Thimerosal- and Hg^{2+} -induced volatilization of $^{203}\text{Hg}^0$ from $^{203}\text{Hg}^{2+}$. *S. aureus* strains RN4 (○), 1123 (●), and 1255 (△) were grown in nutrient broth and induced by addition of the indicated concentration of thimerosal (left) or Hg^{2+} (right). After 1 h of further growth, the cells were harvested by centrifugation and suspended in buffer (17), and the rate of volatilization of mercury from $5 \mu\text{M}$ $^{203}\text{Hg}^{2+}$ was measured as previously reported (17, 28).

summary of these resistance patterns plus other information obtained with the four new *S. aureus* isolates.

There are two possible explanations for the thimerosal resistance of the new *S. aureus* isolates: either the organomercurial lyase(s) responsible for organomercurial detoxification has an extended substrate range, or merbromin and thimerosal function as more effective inducers of this strictly inducible system (28) with the new isolates. A quantitatively better functioning of thimerosal as an inducer of mercury volatilizing activity (a measure of the mercuric reductase enzyme) was seen with the two new isolates 1123 and 1255 when compared with strain RN4 (Fig. 2), whereas with Hg^{2+} as the inducer, strain RN4 responded with higher activity levels than did the new isolates. The difference in effectiveness as inducer of the mercuric resistance operon seems sufficient to account for the added resistance to thimerosal shown by strains 1123 and 1255 (Fig. 1). Attempts to determine differences between the new *S. aureus* isolates and strains RN4 and U71 with regard to merbromin as an inducer were unsuccessful. With all strains, subtoxic concentrations ($10 \mu\text{M}$ or less) gave maximum induction of mercury volatilization activity (data not shown).

Differences in volatilization activity toward both thimerosal and methylmercuric chloride were seen. Earlier studies showed that the laboratory *S. aureus* strains had no activity toward methylmercury (28). In recent experiments,

cells of strains RN4, RN23, and U71 showed no volatilizing activity when exposed to $[^{14}\text{C}]$ -methylmercury, but cells of strains 1123 and 1255 volatilized ^{14}C (released as CH_4 as shown earlier by gas chromatography; 24) from methylmercury (Fig. 3). Strain 3794 also volatilized ^{14}C from methylmercury (data not shown). Lyso-staphin cell-free lysates showed activities similar to those of intact cells (Fig. 3B). The loss of ^{14}C from methylmercury by cell-free preparations from strain 1123 showed Michaelis-Menten kinetics, with a K_m of $67 \mu\text{M}$ CH_3HgCl (data not shown).

Volatilization of mercury from nonradioactive thimerosal was measured by flameless atomic absorption analysis of mercury left in media. Uninduced cells of the new isolate 1123 volatilized mercury from thimerosal after a delay of about 15 min (Fig. 4A); when previously induced with Hg^{2+} , cells of strain 1123 volatilized mercury from thimerosal without any delay (Fig. 4B). The thimerosal-susceptible strain RN4 detoxified thimerosal more slowly with induced cells (confirming results in reference 28) and essentially not at all with uninduced cells (Fig. 4). The ability of induced cells of strains 1255 and 3794 to volatilize mercury from thimerosal was identical to that of strain 1123 (data not shown). Thus, the thimerosal resistance of these strains is associated with both a difference in thimerosal as an inducer of the mercurial detoxification system (Fig. 2) and a greater activity for thimerosal detoxification (Fig. 4).

DISCUSSION

We attempted to determine sources of mercurials in hospitals that might select for mercurial resistance plasmids, but were informed that the U.S. Environmental Protection Agency had prohibited use of mercurials for most hospital purposes starting in 1971 to 1972 (D. L. Gravens, personal communication). We have not been able to obtain current figures on mercurial usage in Barnes Hospital, but are assured that mercurials are essentially no longer in use. Before 1971, phenylmercury, methylmercury, and thimerosal (all of which are detoxified by enzymes governed by mercurial resistance plasmids [6, 17, 25, 28]) were used at levels of 10 to 30 mg/liter in hospital liquid detergents to inhibit the growth of bacteria (largely pseudomonads) in these products. Similar reduction or elimination of the usage of mercurials occurred in the United Kingdom (R. W. Lacey, personal communication), the German Democratic Republic (W. Witte, personal communication), and Japan. At Jikei University Hospital, mercurial consumption from 1972 to 1977 averaged 2.2 kg of HgCl_2 , 3.8 kg of merbromin, and 150 g of

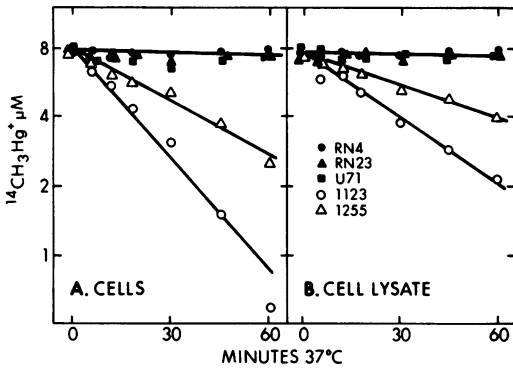


FIG. 3. Volatilization of ^{14}C from $^{14}\text{CH}_3\text{HgCl}$. Thimerosal-resistant strains 1123 (○), and 1255 (△) and thimerosal-susceptible mercuric-resistant strains RN4 (●), RN23 (▲), and U71 (■) were grown to mid-log phase in nutrient broth and induced with $1\ \mu\text{M}\ \text{Hg}^{2+}$ for 30 min followed by $5\ \mu\text{M}\ \text{Hg}^{2+}$ for 30 min. After centrifugation and suspension in phosphate buffer, cell lysates (100 μl) were made by treating the cells with 55 U of lysostaphin. DNase was used to reduce the viscosity of the lysate. The cells (A) and cell lysates (B) were then tested with $8\ \mu\text{M}\ ^{14}\text{CH}_3\text{HgCl}$ in complete assay buffer (17) containing cysteine and NADPH.

thimerosal per year. After 1976 to 1977, the use of mercurials was discontinued.

In the first report of mercurial-resistant *S. aureus*, Moore (12) found that mercuric salts used to disinfect suture catgut would inhibit the growth of *S. aureus* from other sources, but not those isolated from surgical wounds. Moore (12) attributed mercurial resistance to selection by mercurial usage. Tynecka (26) confirmed that mercuric resistance occurred most frequently in phage types of *S. aureus* that were commonly associated with human disease but was unable to establish a direct relationship between mercuric resistance and pathogenicity in mice. Hall (8, 9) considered that mercurial diuretics might be the major source of selection for mercurial resistance in hospitals since mercurial-resistant *S. aureus* strains were isolated more often from geriatric patients (who were treated with diuretics) and less often from surgical or obstetric patients (8). Hall (9) also showed a higher incidence of mercuric resistance with *S. aureus* isolated from the nasal passages of factory workers exposed to mercury salts than from a control group. Summers et al. (22) determined frequency of mercurial resistance in bacteria isolated from hospital sewage and city sewage. The hospital sewage contained a higher frequency of mercuric-resistant isolates among gram-negative bacteria. Since our hypothesis is that selection for mercuric resistance was largely from non-therapeutic hospital usage, then perhaps we

would have found higher frequencies among strains isolated from hospital environments than from those isolated from patients.

R. W. Lacey (personal communication) has questioned the direct selection of mercurial resistance by disinfectants, since hospital disinfectants are not used in nasal passages or on superficial skin wounds (normal habitats for *S. aureus*). Perhaps the critical stage for selection is between habitats rather than during the phase of most elaborate growth. Groves et al. (7) did not find higher frequencies of mercurial-resistant *S. aureus* in isolates from people who had been poisoned by methylmercury. The hypothesis of direct selection by mercurial usage is not proven. Witte and Lacey (personal communications) state that mercurial selection in the hospital would not explain the strong association of mercurial resistance with *S. aureus* from hospital sources and the virtual absence of such resistance from *S. aureus* associated with animal infections (11, 31). Both of these researchers emphasize the association of mercurial resistance with particular phage types and assume that this association must have a causal basis or be a relic from selection in ancient times rather than from current selection in the hospitals. Each of our four new mercuric-resistant *S. aureus* isolates was of a different phage type. Multiple antibiotic resistance was also associated with "hospital staph," but again this appeared coincidental and probably based on selection rather than on a common physiological factor leading to both virulence and resistance to antibiotics (8, 10, 15, 26).

If the various heavy metal resistance genes were tightly linked to antibiotic resistance genes, then one would expect them all to behave in a similar manner. This is not the case. The striking difference between hospital and nonhospital *S. aureus* isolates found with mercury (11, 31) has not been seen with other heavy metal resistances. In this study we found a significant drop in recent years in the levels of mercurial resistance in *S. aureus*, *E. coli*, and *K. pneumoniae*, whereas at the same time the frequencies of cadmium and arsenate resistances remained high. Furthermore, if selection for heavy metal resistance genes was due to linkage with antibiotic resistance genes, then one would expect specific resistance patterns to be present. No such patterns were observed (Table 3); each isolate was unique. It had been concluded earlier by Nakahara et al. (10, 13-15) that heavy metal resistances were not tightly linked with specific antibiotic resistances.

The frequencies of mercury resistance found in our current study are lower than those in previous *S. aureus* collections. This may directly reflect the lowered rates of current usage of

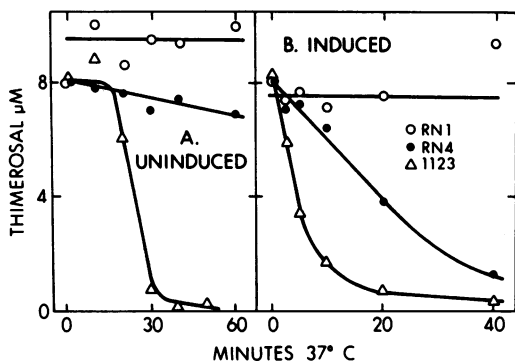


FIG. 4. Volatilization of mercury from thimerosal. Nutrient broth cultures of *S. aureus* strains RN1 (no plasmid, ○), RN4 (plasmid pI524, ●), and 1123 (△) were grown to mid-log phase and divided, and $1 \mu\text{M}$ Hg^{2+} was added to half followed, after 30 min of growth, by an additional $5 \mu\text{M}$ Hg^{2+} . After a subsequent 30 min of growth, the uninduced (A) and induced (B) cells were centrifuged. The induced cells were washed once with nutrient broth, and all six cultures were suspended at 0.75 mg (dry weight)/ml. Thimerosal ($8 \mu\text{M}$) was added, and, during incubation at 37°C with shaking, 0.4-ml samples were "quenched" in 11 ml of a permanganate-sulfuric acid-nitric acid solution (1). After digestion at 55°C for 20 min and overnight at 4°C , the samples were transferred to biochemical oxygen demand bottles for flameless atomic absorption spectroscopy, and chemical reduction and volatilization were carried out according to the manufacturer's (Perkin-Elmer Co.) manual.

mercurials. If so, we can anticipate that levels of mercury resistance will continue to decrease. If mercury resistance on plasmids of *S. aureus* appears in response to direct selection, and we have now begun to guess what the sources of such selection might be, we can ask next what might be the selective forces that maintain high frequencies of cadmium and arsenic resistance in recent hospital collections (Tables 1 and 2).

The new mercuric-resistant *S. aureus* isolates differ slightly from those previously reported (28) since they confer low-level resistances to merbromin, methylmercury, and thimerosal (Fig. 1). These resistances were quantitatively less than the comparable resistances in *E. coli* (Fig. 1; 18, 29) and *P. aeruginosa* (1). We were unable to transfer the new mercuric resistance determinants (or the penicillin resistance determinants of the same strains) into the standard *S. aureus* 8325 strain. Recently, B. Kreiswirth and R. P. Novick (personal communication) succeeded in transferring mercuric resistance from strain 1123 into strain 8325 by protoplast fusion. Thimerosal, merbromin, and penicillin resistances were transferred on a 26-kilobase plasmid along with Hg^{2+} resistance (data not shown).

This preliminary characterization of the new isolates indicates that our earlier report (28) that all *S. aureus* mercuric resistance operons seemed indistinguishable from one another was an oversimplification. With more careful tests and with new clinical isolates, one might hope to find additional patterns of resistances to organomercurials. These in turn might lead to further understanding of how the mercurial resistance determinants are selected and spread within the clinical setting.

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