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Antibiotic Resistance Patterns of Metal-Tolerant Bacteria Isolated from an Estuary

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Estuarine bacteria isolated on metal-containing media were also found to be antibiotic resistant; ampicillin and chloramphenicol were the antibiotics to which resistance was most common. Patterns of antibiotic resistance were found associated with a variety of taxa.

Heavy-metal resistance in bacteria has been shown to be associated with single- or multiple-drug resistance (2, 4, 6, 8). Studies involving *Staphylococcus aureus* strains have clearly demonstrated a correlation between resistance to penicillin, erythromycin, and tetracycline and tolerance to mercury, lead, cadmium, and zinc (2, 8). Similar resistance patterns exist for strains of *Escherichia coli, Salmonella typhimurium,* and *Pseudomonas aeruginosa* (10, 11). The reports published to date, however, concern clinical isolates, although antibiotic- and metal-tolerant bacteria have been found to occur in domestic sewage (11). This study describes antibiotic resistance patterns of metal-tolerant bacteria isolated from an estuary.

Two hundred and thirty strains of heavy-metal-tolerant bacteria were isolated from surface water and sediment samples collected on 8 and 9 December 1975 from Colgate Creek in Baltimore Harbor and Chesapeake Beach, the latter being a comparatively unpolluted site in the Upper Chesapeake Bay. Seventy-one strains from an earlier study of heavy-metal resistance in estuarine bacteria were also included. Procedures used for collection of samples and for isolation, maintenance, and identification of the strains have been described (1). Reference strains of *E. coli* (ATCC 25922) and *P. aeruginosa* (ATCC 27853), recommended for use in antibiotic susceptibility testing (1a), were included in the study.

Pure cultures maintained on Upper Bay yeast extract agar (0.5% [wt/vol] NaCl, 0.15% [wt/vol] $MgSO_4 \cdot 7H_2O$, 0.016% [wt/vol] KCl, 0.1% [wt/vol] proteose peptone [Difco], 0.1% [wt/vol] yeast extract, 2% Difco agar [wt/vol], pH 7.2) were transferred to fresh Upper Bay yeast extract broth, incubated overnight at 25°C, and inoculated onto Mueller-Hinton agar (Difco) with sterile cotton swabs. The inoculated agar plates were left at room temperature to dry before antibiotic Sensi-Discs were applied, after which the plates were incubated for 24 h at 25°C. The antibiotics tested included ampicillin (10 μg), chloramphenicol (5 μg), gentamicin (10 μg), kanamycin (10 μg), streptomycin (10 μg), and tetracycline (30 μg). With the exception of kanamycin, which was obtained from Difco Laboratories, Detroit, Mich., the antibiotic disks were from Bioquest (Division of Becton, Dickinson & Co.) Cockeysville, Md. Susceptibility or resistance of an isolate to an antibiotic was determined by measuring the size of the zone of inhibition. For purposes of this study, an isolate was recorded to be resistant if growth occurred up to the edge of the disk on the agar plate. Antibiotic resistance was recorded for strains capable of growth on Mueller-Hinton agar.

Metal-tolerant bacteria, i.e., those bacteria capable of growth in the presence of metals, were readily recovered from Chesapeake Bay, and these bacteria also demonstrated resistance to antibiotics, including ampicillin, chloramphenicol, gentamicin, kanamycin, streptomycin, and tetracycline (Table 1). Multiple-antibiotic resistance, i.e., resistance to two or more antibiotics (usually ampicillin and chloramphenicol) was found to occur in 16% (39 strains) of the strains tested. Resistance to ampicillin (24%) and to chloramphenicol (32%) were relatively common, whereas few strains were resistant to gentamicin (1%), kanamycin (2%), or tetracycline (3%).

Strains isolated from media containing cobalt, lead, mercury, and molybdenum demonstrated resistance to ampicillin and chloramphenicol, whereas only a few of the strains isolated from media containing lead or molybdenum were resistant to gentamicin (Table 1). Furthermore, most of the strains in taxa comprising the metal-tolerant strains, as reported previously (1), were resistant to one or more antibiotics (see Table 2). Although multiple heavy-metal tolerance was observed in some strains, the antibiotic resistance patterns of these

strains were more restricted (Table 3). Only two phena, *Pseudomonas* (phenon 1d) and a group of unidentified gram-negative rods (phenon 5), were susceptible to all of the antibiotics tested in the study. Seven phena, including *Pseudomonas* (phenon 1c), *Erwinia carotovora* (phenon 3), coryneforms (phenon 7), *Mycobacterium* (phenon 8), and *Bacillus* (phena 10, 11, and 12), were resistant to only one or two of the antibiotics. Of the remaining seven phena, strains of *Pseudomonas maltophilia* (phenon 2) demonstrated resistance to all antibiotics tested. It is noteworthy that *P. maltophilia*, numerically one of the largest clusters of metal-tolerant bacteria recovered in the study, also showed resistance to all of the heavy metals tested (Table 2). Similarly, strains of *Pseudomonas fluorescens* (phenon 1a) were tolerant to heavy metals (Table 2) and resistant to ampicillin and chloramphenicol (Table 3).

Pseudomonas alcaligenes (phenon 1b), unidentified gram-negative, yellow chromogens (phenon 4b), and coryneforms (phenon 6) demonstrated resistance to ampicillin, chloramphenicol, and streptomycin. *Bacillus megaterium* (phenon 9) and yellow chromogens (phenon 4c), both found to be numerically dominant of the metal-tolerant organisms isolated from Chesapeake Bay water and sediment, were observed to be susceptible only to gentamicin and streptomycin, and to gentamicin, respectively. Few strains of these taxa were re-

TABLE 1. *Antibiotic resistance of metal-tolerant bacteria*

Isolation medium	No. of strains examined	% Resistant to:					
		Ampicillin	Chloramphenicol	Gentamicin	Kanamycin	Streptomycin	Tetracycline
Cobalt	52 (21)[a]	33	67	0	5	14	5
Lead	82 (81)	10	21	1	0	4	3
Mercury	60 (57)	56	25	0	4	7	5
Molybdenum	36 (33)	12	36	3	3	15	0
Control	71 (56)	18	41	0	0	25	2
Total no. of strains	301 (248)	24	32	1	2	10	3

[a] Number in parentheses refers to number of strains growing on Mueller-Hinton medium and, thereby, tested for antibiotic resistance.

TABLE 2. *Metal resistance of genera identified during the study*

Phenon[a]	Presumptive identification	No. of strains	% Resistant[b] to:					
			Cd^{2+}	Cr^{3+}	Co^{2+}	Pb^{2+}	Hg^{2+}	MoO_4^{2-}
1a	*Pseudomonas fluorescens*	8 (8)[c]	100	88	87	87	75	100
1b	*P. alcaligenes*	51 (50)	16	68	39	100	82	100
1c	*Pseudomonas* spp.	3 (3)	100	100	100	100	33	100
1d	*Pseudomonas* spp.	2 (2)	100	100	50	100	100	100
2	*P. maltophilia*	43 (39)	2	32	9	88	88	98
3	*Erwinia carotovora*	2 (2)	50	100	0	100	50	100
4a	Unidentified yellow chromogens (gram-negative rods)	52 (15)	48	60	73	56	60	76
4b	Unidentified yellow chromogens (gram-negative rods)	30 (21)	20	60	0	30	3	50
5	Unidentified gram-negative rods	2 (2)	50	0	100	100	50	100
6	Coryneforms	19 (18)	10	42	37	100	100	100
7	Coryneforms	7 (7)	0	57	14	100	100	100
8	*Mycobacterium* spp.	12 (12)	0	75	17	100	33	83
9	*Bacillus megaterium*	53 (53)	38	91	58	100	91	100
10	*Bacillus* spp.	2 (2)	50	50	100	100	100	100
11	*Bacillus* spp.	6 (6)	17	33	17	100	83	100
12	*Bacillus* spp.	2 (2)	50	50	50	100	100	100

[a] Identification of the metal-tolerant bacteria was achieved using numerical taxonomy, the results of which are to be published separately.

[b] Concentration of heavy metals used in the study were: 100 μg/g (Cd^{2+}, Cr^{3+}, Co^{2+}, Pb^{2+}, and MoO_4^{2-}) and 10 μg/g (Hg^{2+}).

[c] Numbers in parentheses refer to the number of strains capable of growth on Mueller-Hinton medium and included in the study.

TABLE 3. *Antibiotic resistance of generic groupings of strains of metal-tolerant bacteria examined in this study*

Phenon	Presumptive identification	No. of strains	% Resistant to:					
			Ampicillin	Chloramphenicol	Gentamicin	Kanamycin	Streptomycin	Tetracycline
1a	*Pseudomonas fluorescens*	8 (8)[a]	100	100	0	0	14	13
1b	*P. alcaligenes*	51 (50)	20	55	0	0	7	0
1c	*Pseudomonas* spp.	3 (3)	0	67	0	0	0	0
1d	*Pseudomonas* spp.	2 (2)	0	0	0	0	0	0
2	*P. maltophilia*	43 (39)	31	21	3	5	5	3
3	*Erwinia carotovora*	2 (2)	50	0	0	0	0	0
4a	Unidentified yellow chromogens	52 (15)	27	40	0	7	17	7
4b	Unidentified yellow chromogens	30 (21)	10	29	0	0	38	0
5	Unidentified gram-negative rods	2 (2)	0	0	0	0	0	0
6	Coryneforms	19 (18)	17	22	0	0	8	0
7	Coryneforms	7 (7)	14	0	0	0	0	0
8	*Mycobacterium* spp.	12 (12)	0	75	0	0	75	0
9	*Bacillus megaterium*	53 (53)	26	11	0	2	0	6
10	*Bacillus* spp.	2 (2)	50	0	0	0	0	0
11	*Bacillus* spp.	6 (6)	83	16	0	0	0	0
12	*Bacillus* spp.	2 (2)	50	0	0	0	50	0

[a] Number in parentheses refers to the number of strains capable of growth on Mueller-Hinton medium and hence examined in this study.

sistant to kanamycin and tetracycline. Because antibiotic resistance is not a stable characteristic, in the sense that the occurrence of antibiotic resistance is greatly variable within bacterial taxa, it is not useful, in general, for identification of taxa. Furthermore, multiple-antibiotic resistance, including resistance to ampicillin and chloramphenicol, was restricted to only 16% (39) of the metal-tolerant bacteria and was represented among a wide assortment of taxa.

In conclusion, bacteria resistant to both antibiotics and heavy metals can readily be isolated from the natural environment, with greater abundance noted for polluted sites, such as Colgate Creek in Baltimore Harbor. Clinically important isolates, such as *Enterobacteriaceae* strains (9–11) and *S. aureus* (4, 8), have been extensively surveyed, but very few data have been gathered for environmental samples (3, 7). Therefore, from results of this study, it is concluded that antibiotic-resistant bacteria are widely distributed in the estuarine environment and that there is an association of antibiotic resistance with metal resistance in estuarine bacteria, other than *Enterobacteriaceae* and *S. aureus*.

Although both antibiotic and metal resistance factors are known to be located on plasmids (4, 10), it has been suggested that plasmids determining metal tolerance may differ from those conferring antibiotic resistance (5). Work is under way in our laboratory to elucidate the role of plasmids in metal and antibiotic resistance of bacteria native to the estuarine environment.

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LITERATURE CITED

1. **Austin, B., D. A. Allen, A. L. Mills, and R. R. Colwell.** 1977. Numerical Toxonomy of heavy metal tolerant bacteria isolated from an estuary. Can. J. Microbiol. 23:1433–1447.
1a. **Bauer, A. W., W. M. M. Kirby, J. C. Sherris, and M. Twick.** 1966. Antibiotic susceptibility testing by a standardized single disk method. Am. J. Clin. Pathol. 45:493–496.
2. **Hall, B. M.** 1970. Distribution of mercury resistance among *Staphylococcus aureus* isolated from a hospital community. J. Hyg. 68:111–119.
3. **Koditschek, L.** 1976. Antimicrobial resistant bacteria in the New York Bight. Am. Soc. Limnol. Oceanogr. Spec. Symp. 2:383–393.
4. **Kondo, I., T. Ishikawa, and H. Nakahara.** 1974. Mercury and cadmium resistances mediated by the penicillinase plasmid in *Staphylococcus aureus*. J. Bacteriol. 117:1–7.
5. **Kondo, I., T. Ishikawa, and H. Nakahara.** 1976. Heavy metal resistance in *Staphylococcus aureus*. Zentralbl. Bakteriol. Parasitenkd. Infektionskrank. Hyg. Abt. 1 Suppl. 5:217–230.
6. **Moore, B.** 1960. A new screen test and selective medium for the rapid detection of epidemic strains of *Staphylococcus aureus*. Lancet ii:453.
7. **Morgan, R. C., P. Guerry, and R. R. Colwell.** 1976. Antibiotic resistant bacteria in Chesapeake Bay. Chesapeake Sci. 17:216–219.
8. **Novick, R. P., and C. Roth.** 1968. Plasmid-linked resistance to inorganic salts in *Staphylococcus aureus*. J. Bacteriol. 95:1335–1342.
9. **Pickett, A. W., and A. C. R. Dean.** 1976. Antibiotic resistance of cadmium- and zinc-tolerant strains of *Klebsiella (Aerobacter) aerogenes* growing in glucose-limited chemostats. Microbios Lett. 1:165–167.
10. **Smith, D. H.** 1967. R factors mediate resistance to mercury, nickel and cobalt. Science 156:1114–1116.
11. **Varma, M. M., W. A. Thomas, and C. Prasad.** 1976. Resistance to inorganic salts and antibiotics among sewage-borne Enterobacteriacease and Achromobacteriaceae. J. Appl. Bacteriol. 41:347–349.