

Antibiotic Resistance Patterns of Gram-Negative Bacteria Isolated from Environmental Sources†

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A total of 2,445 gram-negative bacteria belonging to fecal coliform, *Pseudomonas*, *Moraxella*, *Acinetobacter*, and *Flavobacterium-Cytophaga* groups were isolated from the rivers and bay of Tillamook, Oregon, and their resistances to chloramphenicol (25 µg/ml), streptomycin (10 µg/ml), ampicillin (10 µg/ml), tetracycline (25 µg/ml), chlortetracycline (25 µg/ml), oxytetracycline (25 µg/ml), neomycin (50 µg/ml), nitrofurazone (12.5 µg/ml), nalidixic acid (25 µg/ml), kanamycin (25 µg/ml), and penicillin G (10 IU/ml) were determined. Among fecal coliforms the bay isolates showed greater resistance to antibiotics than those from tributaries or surface runoff. No such well-defined difference was found among other bacterial groups. The antibiotic resistance patterns of gram-negative bacteria from different sources correlated well, perhaps indicating their common origin. The antibiotic resistance patterns of gram-negative bacteria of different genera also correlated well, perhaps indicating that bacteria which share a common environment also share a common mode for developing antibiotic resistance.

Grabow et al. (8) reviewed the public health implications of drug-resistant coliforms in water supplies, and suggested that the prevalence of these drug-resistant bacteria requires reevaluation of water quality standards as well as more advanced purification of sewage prior to discharge into the environment. The ability of various gram-negative bacteria to produce disease is well known (14), and the ability of resistance factor-containing (R⁺) bacteria, particularly *Escherichia coli*, to transfer drug resistance is similarly well known (19). Presumably, drug resistance transfer is most likely to occur in the alimentary canal following ingestion of drug-resistant organisms (8), but resistance transfer in wounds has also been reported (13).

The antibiotic resistances of gram-negative bacteria have been determined largely from clinical material. Several studies have described the antibiotic resistance of clinical isolates of *Moraxella* spp. (17, 25), *Acinetobacter* spp. (16, 17, 18), and *Flavobacterium* spp. (1, 12, 17, 25). The antibiotic resistance of clinical isolates of various *Pseudomonas* spp., particularly *P. aeruginosa*, has also received considerable attention. With the exception of the large number of studies that have dealt with the antibiotic resistance of fecal

coliform organisms isolated from nature, little is known about the antibiotic resistance patterns of gram-negative bacteria that occur in the environment. Breuil et al. (4) reported the antibiotic sensitivity of a single *Acinetobacter* spp. isolated from river sediment. Tunstall and Gowlan (23) reported the antibiotic susceptibility of 72 marine *Pseudomonas* spp. Most other studies have used antibiotic sensitivity as a taxonomic tool (5, 24).

The presence in waterways of both potentially pathogenic gram-negative bacteria and fecal coliforms containing transferable R-factors raises the question of whether resistance transfer may actually occur in streams, rivers, bays, and other waterways. Harsh environmental conditions in these areas would seem to minimize the likelihood of transfer but certain microenvironments may provide conditions where resistance transfer could occur.

This study was undertaken to accomplish two goals. The first was to provide detailed descriptive information about the antibiotic resistances of gram-negative bacteria isolated from waterways, and the second was to explore whether antibiotic resistance patterns among different genera vary in a systematic or random manner.

MATERIALS AND METHODS

Sampling. Samples were taken monthly during January, February, and March 1976 from each of four

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tributaries which enter Tillamook Bay, Oregon, and from the bay itself. Bay samples were collected approximately 1.5 miles offshore. Rough sea conditions prevented the collection of a bay sample in February. In February and March, water samples were taken directly from pasture areas adjacent to river sampling sites. Samples were collected during an outgoing tide in 1-gallon (ca. 3.8-liter) sterile Nalgene bottles by directly dipping the bottles into the surface of the water as described in *Recommended Procedures for the Examination of Sea Water and Shellfish* (2). Samples were immediately placed in insulated containers and transported to the laboratory. Microbiological analysis commenced within 2 h after the last sample was collected.

Isolation procedures. Fecal coliform organisms were isolated by using Millipore HAWG 047SO membrane filters and Difco mFc medium (2). Fecal coliform colonies on the filter were transferred with sterile toothpicks to 100-mm petri plates containing tryptone-peptone extract (TPE) agar (10). Colonies were transferred to this plate in a pattern that permitted the subsequent use of a 30-colony-per-plate nichrome wire stab replicator (11). Gram-negative bacilli were isolated by spread-plating water samples onto TPE agar and incubating for 48 h at 25°C. These colonies were then transferred to 100-mm petri plates in the manner described above.

Microbial identification. In accordance with a standard procedure (2), fecal coliform organisms were those which grew on mFc medium with characteristic blue color after 24 h of incubation at $44.5 \pm 0.2^\circ\text{C}$. Gram-negative bacteria were identified by using a classification scheme previously reported (11).

Antibiotic resistance determination. TPE agar was used as the basal medium to determine the antibiotic resistances of fecal coliform organisms. Antibiotics were mixed with molten agar at approximately 45°C or were first diluted in distilled water and then added to the molten agar. Antibiotics and their concentrations employed in this investigation were as follows: chloramphenicol (Parke-Davis), 25 µg/ml; streptomycin (Eli Lilly), 10 µg/ml; ampicillin (Bristol), 10 µg/ml; tetracycline (Lederle), 25 µg/ml; chlortetracycline (Lederle), 25 µg/ml; oxytetracycline (Pfizer), 25 µg/ml; neomycin (Upjohn), 50 µg/ml; nitrofurazone (Eaton), 12.5 µg/ml; nalidixic acid (Calbiochem), 25 µg/ml; kanamycin (Bristol), 25 µg/ml; and procaine penicillin G (Squibb), 10 IU/ml.

The 24-h cultures of fecal coliforms were transferred from TPE agar plates with a 30-colony nichrome wire stab replicator to the antibiotic-containing media. Eleven antibiotic plates and one control plate containing no antibiotic were inoculated consecutively. This method allows slight differences in amount of inoculum. However, the control plate was inoculated last to confirm successful inoculation of preceding plates. Plates were incubated at 37°C for approximately 24 h, and the drug resistance was determined. An organism was considered resistant to an antibiotic only if it grew as well on the antibiotic plate as on the control plate. Any sign of growth inhibition was scored as sensitivity to that antibiotic. This meant that "resistance" was very strictly defined so that no organism with any sign of sensitivity would be classified as resistant.

The antibiotic resistance of gram-negative bacteria was determined in the same manner, except incubation was at 25°C for 48 h.

Correlation. Correlation values were determined by using the following formula:

$$\text{Correlation} = \sum_{k=1}^n (Y_{ik} - \bar{Y}_i)(Y_{jk} - \bar{Y}_j)/(n-1)S_iS_j,$$

where Y_{ik} is the k th observation on variable i ; Y_{jk} is the k th observation on variable j ; \bar{Y}_i is the average of variable i ; \bar{Y}_j is the average of variable j ; n is the number of observations; S_i is the standard deviation of variable i ; and S_j is the standard deviation of variable j .

RESULTS AND DISCUSSION

A total of 2,763 bacterial colonies were isolated, identified to the genus level, and examined for antibiotic resistance. Of these, 2,445 were fecal coliforms, *Pseudomonas*, *Moraxella*, *Acinetobacter*, or *Flavobacterium-Cytophaga*. Table 1 presents the antibiotic resistances of these bacteria. The remaining isolates were either unidentified or belonged to genera not isolated frequently enough to provide meaningful statistical information. Those were *Aeromonas*, *Bacillus*, *Proteus*, *Arthrobacter*, *Lactobacillus*, *Klebsiella*, *Plesiomonas*, *Pectobacterium*, *Chromobacterium*, *Serratia*, *Enterobacter*, *Staphylococcus*, and *Micrococcus*.

Table 1 lists the microorganisms separately by sources, i.e., pasture, tributary, and bay. An examination of Table 1 reveals that, as expected, there are wide differences among bacterial groups in the percentage of resistance of particular antibiotics. This is illustrated by the resistance level of pasture fecal coliform isolates to ampicillin (2%) and pasture *Pseudomonas* isolates to the same antibiotic (90%). Similarly, and again as expected, within the same bacterial group the percentage of resistance to different antibiotics differs greatly. For example, among bay *Flavobacterium-Cytophaga* isolates, none was resistant to tetracycline and chlortetracycline, whereas 82% were resistant to neomycin.

An examination of antibiotic resistance of pasture, tributary, and bay fecal coliform isolates revealed that the percentage of resistant isolates was consistently higher in tributaries than pastures and higher still in the bay than in tributaries. Exceptions included only chloramphenicol resistance, which was 0% from all sources, penicillin G resistance, which was 100% in both pastures and tributaries, and nitrofurazone, nalidixic acid, and penicillin resistances, which were slightly higher in tributary isolates than in bay isolates.

The data suggest that antibiotic-resistant fecal coliforms may survive better than sensitive

All sources	32	22	79	0	20	21	6	86	12	7	85
<i>Pseudomonas</i> spp. (658)	(212)	(144)	(519)	(2)	(130)	(136)	(41)	(564)	(82)	(44)	(561)
<i>Moraxella</i> spp. (71)	14	45	66	3	30	24	17	89	4	11	72
<i>Acinetobacter</i> spp. (410)	6	(32)	(47)	(2)	(21)	(17)	(12)	(63)	(3)	(8)	(51)
<i>Flavobacterium-Cytophaga</i> spp. (275)	(24)	(72)	(205)	(3)	(72)	(67)	(21)	(353)	(23)	(24)	(240)
	6	46	44	1	10	12	60	59	3	68	45
	(17)	(127)	(122)	(2)	(28)	(34)	(164)	(161)	(7)	(188)	(124)
Fecal coliforms (1,031)	0	16	13	17	16	19	7	97	1	8	100
	(0)	(169)	(131)	(173)	(168)	(191)	(74)	(1,004)	(10)	(81)	(1,030)

^a Parentheses indicate total isolates examined.

^b Cm, Chloramphenicol; Sm, streptomycin; Ap, ampicillin; Tc, tetracycline; Ct, chlortetracycline; Ot, oxytetracycline; Nm, neomycin; Ni, nitrofurazone; Na, nalidixic acid; Km, kanamycin; Pc, penicillin. Parentheses indicate number of isolates resistant.

organisms in surface waters. This may be due to conditions similar to those described by Grabow and co-workers (6, 7), who suggested that R-factor-mediated antibiotic resistance increased survival ability of the coliform bacteria. Anderson (3), however, suggested that R-factor-mediated antibiotic resistance may reduce survival ability in *E. coli*, and Smith et al. (21) indicated that R-factor-mediated antibiotic resistance had no effect on *E. coli* survival. In this regard, the resistance data presented in Table 1, based on a large number of fecal coliform isolates, seem to suggest that resistance to antibiotics is related to the survival potential of the fecal coliforms.

To confirm that the antibiotic resistance was R-factor mediated, a conjugation experiment was conducted between 50 randomly selected ampicillin-, streptomycin-, and tetracycline-resistant fecal coliform isolates and an *E. coli* K-12 recipient (W3110, F⁻, nalidixic acid resistant). When plated on streptomycin-nalidixic acid agar, 73% of the recipient cells were able to grow and, among them, 21% were also resistant to ampicillin and 9% to tetracycline. Furthermore, approximately 30% of the isolates lost resistance to one or more antibiotics during 6 months of storage at 5°C.

An examination of Table 1 also reveals that, in sharp contrast to fecal coliform resistances, resistance levels in bay isolates of all other gram-negative bacteria were lower than those of tributary isolates (exceptions were nalidixic acid resistance in *Pseudomonas*, chloramphenicol resistance in *Acinetobacter*, and neomycin and kanamycin resistance in *Flavobacterium-Cytophaga*). This may indicate a decreased survival ability of antibiotic-resistant organisms in these genera, or that some of the bay isolates of these genera were not contributed by the tributaries but, rather, came from other sources. There was, however, no similar trend in antibiotic resistance levels when pasture and tributary isolates were compared; the resistance levels to about half the antibiotics increased, and those to the other half decreased. Any generalization about the effect of antibiotic resistance on survival ability may, therefore, be unjustified for these genera.

This information alone does not address an important question. Do the antibiotic resistance patterns vary in a systematic or nonsystematic way? Table 2, an attempt to partially answer this question, is a correlation matrix of antibiotic resistances of the bacterial groups from all sources and from each individual source; i.e., it displays the correlations among the columns in Table 1. A high correlation indicates systematic variation between antibiotic resistance patterns, and a low correlation indicates nonsystematic

variation between antibiotic resistance patterns. Table 2 demonstrates extremely high correlations among bacteria of the same genus isolated from different sources. For example, the correlation between pasture fecal coliform isolates and tributary fecal coliform isolates was 0.99. Similarly, the correlation between tributary fecal coliform isolates and bay fecal coliform isolates was 0.99. The correlation between pasture *Pseudomonas* isolates and tributary *Pseudomonas* isolates was 0.99, and between tributary *Pseudomonas* and bay *Pseudomonas* isolates was 0.96. Correlation between pasture *Moraxella* isolates and tributary *Moraxella* isolates was 0.96, and between tributary *Moraxella* isolates and bay *Moraxella* isolates it was 0.93. The same comparison shows that *Acinetobacter* yielded a pasture-tributary correlation of 0.95 and a tributary-bay correlation of 0.98. Lower correlations of 0.85 and 0.68 were found for, respectively, pasture-tributary and tributary-bay isolates of *Flavobacterium-Cytophaga*. These results strongly suggest that the antibiotic resistance patterns in these bacterial groups are very similar, perhaps indicating similar mechanisms for the development of this resistance, and also that bay isolates were contributed by the tributaries and that, likewise, tributary isolates were contributed by runoff from the pastures.

Table 2 also demonstrates very high correlations between antibiotic resistance patterns from different bacterial groups. For example, a comparison of correlations between different bacterial groups isolated from pastures reveals that the lowest correlation was between fecal coliforms and *Flavobacterium-Cytophaga* (0.64) and the highest correlation was between *Acinetobacter* and *Moraxella* (0.94). For tributary isolates, the lowest correlation was between *Flavobacterium-Cytophaga* and *Pseudomonas* (0.43) and the highest was between *Acinetobacter* and *Pseudomonas* (0.96). For bay isolates the lowest correlation was between *Flavobacterium-Cytophaga* and *Pseudomonas* (-0.24), and the highest was between *Acinetobacter* and *Pseudomonas* (0.97). For isolates pooled from all sources, the lowest correlation was between *Flavobacterium-Cytophaga* and *Pseudomonas* (0.35), and the highest was between *Acinetobacter* and *Moraxella* (0.96). It should be noted here that, if *Flavobacterium-Cytophaga* isolates were excluded, the "lowest correlation" value would in all cases increase, and, in fact, no comparison would yield a correlation less than 0.70. Thus the information in Table 2 indicates that, with the possible exception of *Flavobacterium-Cytophaga* isolates, the antibiotic resistances of these bacteria are not random but are, rather,

systematic in their occurrence. This could result from similar exposure to antibiotics by all the bacterial groups, and, hence, similar selection pressure being applied to each group. This seems unlikely since, except for the fecal coliforms, most of these bacteria are presumably of soil origin. Another possible explanation is that these bacterial groups have similar chromosomal genetic information which codes for antibiotic resistance. Although possible, it seems unlikely that these different bacterial groups would each evolve to have such similar chromosomal material. More likely is the possibility that some interaction occurs between these different bacteria, which produces these similar antibiotic resistance patterns. Conceivably, a sharing of a pool of R-factor plasmids among these bacterial groups cohabitating a common environment could account for this similarity. Resistance-factor transfer among *E. coli* and *Pseudomonas* is known (15), and it has been demonstrated among *Acinetobacter* (22). The in vivo transposition and insertion of genetic material among plasmids may have a role in the epidemiology of this antibiotic resistance. Rubens et al. (20) and Heffron et al. (9) suggest that this mechanism probably plays a central role in the development of plasmid-mediated antibiotic resistance.

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