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Frequency of Tetracycline Resistance Determinant Classes Among Lactose-Fermenting Coliforms

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Using colony hybridization techniques and DNA probes derived from four distinct tetracycline resistance determinants, we have examined the frequency of these determinants among 225 lactose-fermenting coliforms isolated from fecal samples of both humans and animals. The class B, or Tn*10*-type determinant, occurred most frequently at 73.3%, followed by class A (on RP1) at 21.7%, and class C (on pSC101) at 8%; 3.5% of isolates harbored two of these determinants. Hybridization to class D, carried by plasmid RA1, was not found among any of the isolates. One isolate failed to hybridize to any of the probes and represents a fifth class of determinant. No dramatic differences were observed in the frequencies of these determinants among four populations examined: hospital, urban, rural, and laboratory. At low stringency conditions of hybridization we were able to demonstrate cross-hybridization of determinant A with class C DNA and limited reaction with class B DNA, but no reaction with class D DNA.

Tetracycline resistance, commonly specified by plasmids, is widely disseminated among various bacterial species and is expressed at different levels of resistance (11, 12). We and others have used the tetracycline analogs chelocardin and minocycline to demonstrate phenotypic differences among strains carrying tetracycline resistance (12, 13). Such evidence strongly suggested genetic heterogeneity of these determinants, and this was later confirmed by DNA-DNA hybridization with unique probes for three different phenotypic resistance determinants (12). Subsequent evidence has demonstrated an even greater heterogeneity among tetracycline resistance determinants; there appears to be no detectable homology between those found in gram-negative and gram-positive species (3, 14). Recently, we have developed a specific probe for the tetracycline determinant classified as class D. Using this and probes for the other three determinants, we examined the frequency of classes A through D among 225 tetracycline-resistant, lactose-fermenting coliforms isolated from 199 fecal samples of human and animal donors. All but one of the isolates hybridized to one or more of the known tetracycline resistance determinants. The results support the uniqueness of the four determinants and demonstrate a marked difference in the frequency of these determinants among fecal coliforms. This study also provided evidence for the presence of one more determinant class among the naturally occurring lactose-positive coliforms.

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MATERIALS AND METHODS

Bacterial strains. (i) Wild-type strains. All wild-type coliforms were tetracycline-resistant isolates recovered during 1978 through 1980 from fecal specimens obtained from humans or animals in five different populations: hospital patients, laboratory workers in the greater Boston area, urban dwellers (consisting of first-year medical students of Tufts and Harvard Universities), and a rural population of humans and animals dwelling in the town of Sherburne, Mass. (25 miles west of Boston). All specimens consisted of rectal or fecal swabs collected on Culturettes (Scientific Products Div., McGaw Park, Ill.) and plated with two-dimensional streaking onto MacConkey agar plates. After growth these plates were then replica plated to MacConkey agar containing 10 μg of tetracycline per ml. One or two representative tetracycline-resistant (Tet^r) colonies were picked from each plate, reisolated, and saved for further testing by storage in Penassay agar stabs containing 5 μg of tetracycline per ml. In some instances multiple sampling of the same donor was performed at 2-week intervals. All isolates were subjected to a determination of antibiotic resistance and level of resistance to tetracycline. In cases where multiple isolates were taken from the same donor, each was determined to represent a unique bacterial population before consideration in the final data analyses. This was accomplished by examining colony morphology and determining associated antibiotic resistance patterns as well as the level of resistance to tetracycline.

(ii) Laboratory strains. Four plasmid-bearing strains

were selected or constructed for the isolation and preparation of DNA probes for hybridization: (i) D22-3 contains a pACKC1 derivative bearing the 750-base-pair *Sma*I fragment from the class A determinant on RP1; (ii) D20-7 hosts plasmid pKT007 (a derivative of the ColE1 plasmid pML21) (17) containing the large 4.8-kilobase (kb) *Hin*dIII fragment of the class B tetracycline resistance determinant on Tn*10*; (iii) D20-6 bears plasmid pBR322, from which the class C probe DNA was prepared; (iv) strain D22-2 contains plasmid pSL106, a newly derived construct of the cloning vector pACYC177 and the class D tetracycline resistance determinant from plasmid RA1 (see below).

Determination of antibiotic resistance patterns and tetracycline resistance level. To aid in identifying phenotypically distinct Tet^r populations, the associated antibiotic resistances were identified. The isolates chosen for testing were spotted in a grid pattern onto a MacConkey agar plate. Colonies were grown at 37°C to a small diameter and replica plated to MacConkey agar plates containing the following antibiotics: tetracycline (10 μg/ml), gentamicin (10 μg/ml), kanamycin (10 μg/ml), ampicillin (30 μg/ml), chloramphenicol (25 μg/ml), and cephalothin (30 μg/ml). After overnight incubation at 37°C, the absence or presence of growth was noted.

As an additional aid in distinguishing unique isolates, a crude estimate of the tetracycline resistance level was made. Colonies spotted onto MacConkey agar as above were replica plated onto MacConkey agar plates containing 100, 200, or 300 μg of tetracycline per ml. The estimated inhibitory level was designated as the concentration that completely prevented growth.

Isolation of probe DNA for hybridization. The class A probe was a 750-base-pair *Sma*I fragment from plasmid RP1, which represents about 30% of the entire tetracycline resistance determinant. It was cloned into the *Sma*I site of cloning vector pACKC1, producing recombinant plasmid pSL107 from which the *Sma*I fragment was isolated for use as a probe. DNAs for the class B and C probes were obtained from chloramphenicol-amplified strains D20-7 and D20-6, respectively (5). pKT007 plasmid DNA was restricted with *Hin*dIII endonuclease to obtain a 4.88-kb fragment of the Tn*10* tetracycline resistance determinant. DNA for the class C probe was obtained from pBR322 DNA by isolation of the 928-base-pair fragment excised by *Bst*NI endonuclease. The class D probe consisted of a 3.05-kb *Hin*dIII-*Pst*I fragment excised from plasmid pSL106 (see below).

Plasmid DNAs containing all of the tetracycline resistance determinant probes were purified by centrifugation through cesium chloride (6) before endonuclease treatment. Restriction enzyme assays were performed according to the manufacturer's instructions (New England Biolabs). The desired DNA fragments were separated and then recovered from 1% agarose (Seaplaque; FMC Corp.) either by a butanol extraction (10) or by electroelution for 3 h at 1 W in an ISCO isoelectric concentrator. The DNAs were subsequently precipitated in ethanol and suspended in a 10 mM Tris–1 mM EDTA (pH 8.0) buffer before nick translation.

Nick translation of probe DNA. Nick translation was performed with dATP labeled with either ^{32}P (New England Nuclear Corp.) or ^{35}S (as the [α-thio]triphosphate; gift of New England Nuclear Corp.). The procedure with [^{32}P]dATP was carried out as described previously (12); that with [^{35}S]dATP was performed according to the manufacturer's instructions. The specific activity ranged from 0.4×10^7 to 2.0×10^7 cpm (for ^{35}S) and 1×10^8 to 2×10^8 cpm (for ^{32}P) per μg of DNA.

Preparation of bacterial colonies on filters. Bacterial colonies for hybridization were prepared by inoculating nitrocellulose membrane filters (Schleicher & Schuell Co., BA 85/20) with the tips of 5-μl micropipettes filled with a bacterial suspension freshly grown in L broth. Strains were inoculated in duplicate on each filter, which had been placed on MacConkey agar or on an L broth-containing agar plate with 10 μg of tetracycline per ml. The plates were incubated at 37°C overnight. The filters were treated by the method of Grunstein and Hogness (7), with the exception that colony debris was removed in the last NaCl washes by gentle scraping of the filter surface with blunt-end forceps. Filters were baked at 80°C for 2 h and stored desiccated under vacuum pending hybridization.

Hybridization. Colony hybridization with [^{35}S]dATP was performed by incubation of the filters in a 3-ml reaction mixture consisting of 5× SSPE (1× SSPE = 0.18 M NaCl, 10 mM NaH_2PO_4, 1 mM disodium EDTA, pH 7.0), 0.3% sodium dodecyl sulfate, 100 μg of denatured salmon sperm DNA (type III; Sigma Chemical Co.) per ml, 50% (vol/vol) formamide, and 0.5×10^6 to 1.0×10^6 dpm of denatured, nick-translated probe DNA in 10 mM Tris, 1 mM trisodium EDTA (pH 7.5). The mixture was heat sealed in plastic bags and incubated at 42°C for 20 h. Hybridizations with [^{32}P]dATP were performed as described previously (12). After incubation, the filters were washed four times for 15 min each in 250 ml of 2× SSPE–2% sodium dodecyl sulfate at 45°C with agitation. The filters were air dried overnight and exposed to XAR-5 X-ray film (Eastman Kodak Co.) for 3 to 4 days before processing in a Kodak RP X-Omat processor.

RESULTS

Cloning of the tetracycline resistance determinant on RA1 for class D probe. Isolation of DNA containing the class D determinant was initiated by the selection of a tetracycline-resistant λ-lysogen (strain EO-9) after λ Sam7 infection of a strain containing plasmid RA1 (2). This strain, now carrying the complete resistance determinant incorporated into λ, was temperature induced to produce phage (6), and the isolated and purified DNA was restricted with several endonucleases to identify the size of the inserted RA1 DNA. Analyses of the restriction patterns showed that *Hin*dIII treatment produced only a single change in the λ digestion profile: a new 4.3-kb fragment was detected when compared with the restriction pattern of parent λ DNA (Fig. 1). By digestion and ligation, this fragment was cloned into the single *Hin*dIII site of plasmid pACYC177 (pSL101, Fig. 2). Insertion of the fragment was confirmed by the inactivation of kanamycin resistance in the host vector and

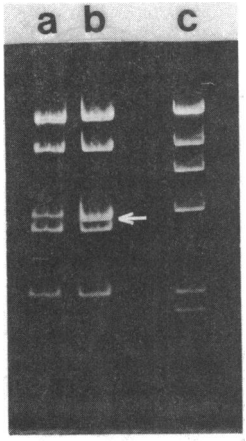

FIG. 1. Agarose gel electrophoresis of *Hin*dIII restriction of (a) λ Sam7 lysogen DNA and (b) the same DNA containing the 4.3-kb fragment (arrow) specifying tetracycline resistance from plasmid RA1. (c) λ DNA *Hin*dIII digest standard (New England Biolabs), from top to bottom: 23, 9.8, 6.6, 4.5, 2.5, and 2.2 kb.

subsequent expression of tetracycline resistance. This plasmid retained the complete inducible tetracycline resistance characteristic of RA1 and demonstrated the insertion of the 4.3-kb *Hin*dIII restriction fragment into the cloning vector. We could further reduce the size of this fragment by treatment with either *Hin*cII or *Pst*I enzymes (Fig. 2). The larger *Hin*dIII-*Hin*cII fragment (3.57 kb, on pSL105) retained inducible high-level resistance. The smaller *Hin*dIII-*Pst*I fragment (3.05 kb) was chosen for the D probe. This was ligated with pACYC177, after restricting the vector with the same enzymes. By selection for tetracycline resistance, we obtained a bacterial clone that contained a plasmid (pSL106) bearing the 2.45-kb pACYC177 fragment and a 3.05-kb fragment (Fig. 2) capable of expressing high-level but noninducible tetracycline resistance.

Hybridization of probes to naturally occurring fecal coliforms. A total of 225 tetracycline-resistant coliforms were isolated from 199 fecal or rectal samples obtained from 127 humans and 25 animals. Examination of all isolates for hybridization to class A, B, C, or D resistance probes revealed that the majority (73.3%) of wild-type, tetracycline-resistant coliforms carried the class B (Tn*10*-type) determinant (Table 1). About one-fifth (21.7%) hybridized with class A, whereas class C occurred less commonly at a level of 8%. None of the isolates hybridized with the class D probe. The distribution of these determinants among the various populations was not notably different; however, the rural human group had a considerably lower frequency of class A determinants (6.3%), and class C determinants were absent from the urban group of medical students. This latter finding is unexplained, since most laboratory workers were also urban dwellers, and they demonstrated the presence of the class C determinant. Upon comparing all persons living in an urban or rural environment, the only difference noted was a decrease in frequency of class C determinants among the urban group accompanied by a relative increase in class A determinants. These differences were not found to be statistically significant.

Eight of the 225 isolates tested were found to hybridize with two of the probes in combinations of A and B (2.2%) or A and C (1.3%). These were found among hospital and urban groups, but not in rural dwellers. Only one isolate (from a laboratory worker) failed to hybridize with any probe in repeated testings.

Although in general only one isolate was tested from each fecal sample, when two from a single sample were examined, it was not unusual to find two different tetracycline resistance classes present. This became more obvious in multiple samples from the same persons where different isolates from the same person showed different tetracycline resistance determinants present over a period of 2 or more weeks.

To investigate the possible relatedness of the four resistance determinants, we examined colony hybridization under less stringent conditions (9). Using labeled class A probe DNA, we noted that the class C and class B determinants cross-hybridized at formamide concentrations of ≤40% (Fig. 3). Using the class B probe, we found no cross-hybridization. The ability to detect cross-hybridization of the B determinant to class A DNA probe, but not the reverse, may relate to the multiple copies of class B determinant on plasmid pKT007 (found in test strain D20-7) and a low copy number of the A determinant in the test strain bearing RP1. The class D determinant demonstrated minimal or no hybridization with either A or B probes, even under conditions of low formamide concentration. The results show more homology of determinant class A with class C than with determinants of classes B and D.

DISCUSSION

Our results demonstrate that the four known tetracycline determinants reside in wild-type coliforms at various frequencies, with that of the Tn*10*-like element as the most common. These findings suggest that gram-negative aerobic intestinal flora, particularly the lactose-fermenting coliforms, serve as a reservoir and possible source of dissemination for these determinants. Other workers examining gram-positive organisms have failed to show detectable homology

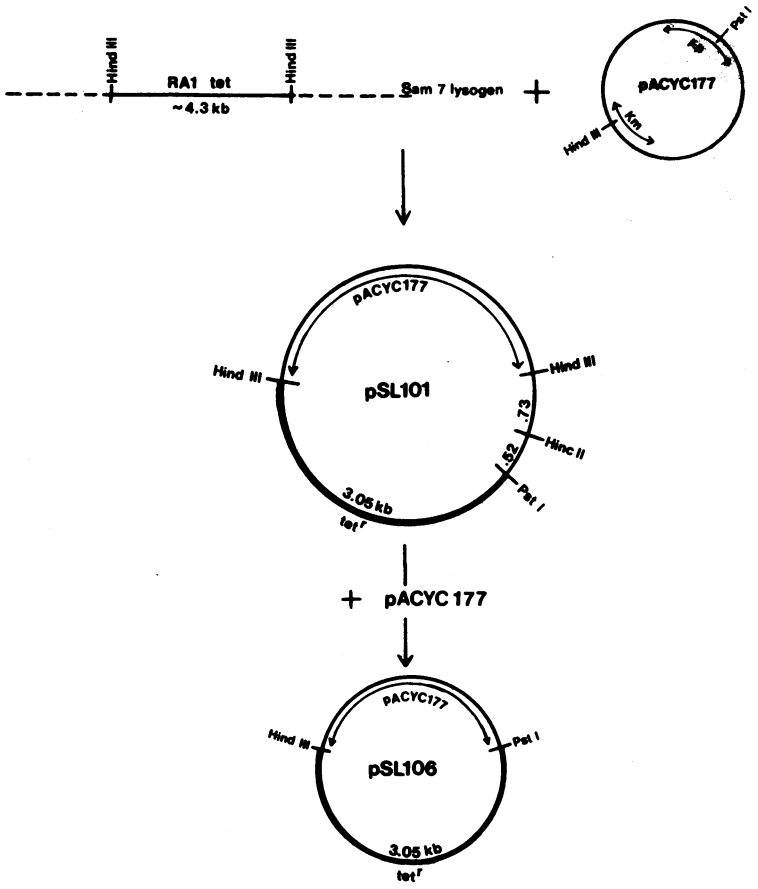

FIG. 2. Construction of pSL106. A 4.3-kb fragment from plasmid RA1 was isolated on a λ Sam7 lysogen (see text). By *Hin*dIII excision and ligation, the fragment was cloned into plasmid pACYC177 (4), producing plasmid pSL101. A 3.05-kb *Hin*dIII-*Pst*I fragment was likewise excised and religated into pACYC177 to produce plasmid pSL106.

between our four probes and determinants in pneumococci (14) and other groups of streptococci (3). We have also failed to demonstrate homologous tetracycline resistance determinants among the gram-positive bacteria we have tested (unpublished data).

Our findings raise speculation as to the origin, dissemination, and stability of resistance determinants in nature. The unequal distribution of the four determinants among the coliform population suggests that the lactose fermenters may not be the original hosts for all these determinants. If other bacterial genera demonstrated a similar distribution pattern, such a conclusion

TABLE 1. Frequency of tetracycline resistance determinant classes

Population	No. of donors	No. of samples	No. of isolates	No. positive (% within group) in determinant class:						
				A	B	C	D	Other	A and B	B and C
Hospital	31	31	31	5 (16.1)	27 (87.1)	2 (6.5)	0	0	2	1
Laboratory	44	80	87	21 (24.1)	58 (66.7)	8 (9.2)	0	1	1	0
Urban	29	29	38	15 (34.5)	25 (72.4)	0 (0)	0	0	2	0
Rural human	23	27	32	2 (6.3)	27 (84.4)	3 (9.4)	0	0	0	0
Rural animal	25	32	37	6 (16.2)	28 (75.7)	5 (13.5)	0	0	0	2
Total	127 humans, 25 animals	199	225	49 (21.7)	165 (73.3)	18 (8)	0	1 (0.4)	5 (2.2)	3 (1.3)

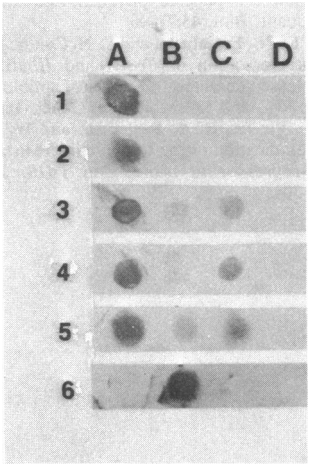

FIG. 3. Hybridization under reduced stringency conditions. Columns A, B, C, and D represent DNA from colonies bearing plasmids with resistance determinants for classes A (plasmid RP1), B (plasmid pKT007), C (plasmid pBR322), and D (plasmid RA1), respectively. Rows 1 through 5 show hybridization with ^{35}S-radiolabeled probe A DNA under various formamide concentrations (from top to bottom): 60%, 50%, 40%, 30%, 20%. Row 6 shows the reaction of radiolabeled probe B DNA with colony DNAs under conditions of 20% formamide.

would not merit consideration. However, examination of other populations has thus far revealed very different frequencies of these determinants. For example, data derived from the testing of small populations of *Pseudomonas* spp. and *Aeromonas* spp. reflect a much higher frequency of the class A determinant (S. B. Levy et al., unpublished data). Likewise, the class B determinant is highly prevalent among *Hemophilus* spp. examined to date (S. B. Levy, A. Buu-Hoi, and B. Marshall, manuscript in preparation; B. Marshall, M. Roberts, A. Smith, and S. B. Levy, manuscript in preparation). However, other factors bearing consideration are that the frequency of a determinant class in a particular species may also be reflected by the relative stability of the determinant or its vector (or both) in the host and the length of time since its emergence. This is exemplified by *Hemophilus* spp., in which the class B determinant is widely disseminated, but has only recently been recognized in this genus (5). Although the class D determinant was not detected in this survey of fecal flora, we have found it in at least one tetracycline-resistant strain of *Vibrio cholerae* (unpublished data). We have also demonstrated that a fifth unidentified determinant occurs rarely among coliform bacteria.

Despite the clear distinction between determinants by DNA-DNA hybridization under stringent conditions, we were able to show cross-hybridization between A, B, and C determinants as we decreased the stringency of the hybridization conditions. Thus a relationship exists between these determinants. However, the lack of reaction with class D DNA suggests much less homology with this determinant. In accord with these findings, from recent DNA sequence data (8, 16), we estimate 74% sequence homology between class A (S. Waters, personal communication) and class C (16) determinants and a 48% homology between classes B and C (8). We have calculated a 41% homology between determinant classes A, B, and C. This information and the fact that the genetic organization of the determinants A, B, and C are similar (1, 15, 18) strongly suggest a common origin for these determinants.

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