SPECIES COMPOSITION AND GROWTH-TEMPERATURE CHARACTERISTICS OF COLIFORMS IN RELATION TO THEIR SOURCES

AKIRA HIRAISHI¹ AND SUSUMU HORIE

Department of Food Science and Technology, Tokyo University of Fisheries, Minato-ku, Tokyo 108, Japan

(Received November 8, 1981)

Species composition and growth-temperature characteristics of coliforms isolated from several types of environmental samples were investigated. It was possible to divide these isolates into eight distinct groups, six species groups and two unidentified, on the basis of their biochemical properties. The isolates included in the unidentified groups, giving IMViC reactions of $--++$ and $-+-+$, were tentatively termed "typical psychrotrophic coliform (TPC)" bacteria because of their ability to grow at 1°. The TPC bacteria and *Citrobacter freundii* constituted the normal coliform flora of mountain soil and stream water samples. In contrast, *Escherichia coli* and *Klebsiella pneumoniae* were major predominant coliforms in polluted river water and sewage samples, the former being most common in human feces samples. In testing the growth response at different temperatures, the isolates of *E. coli* and the TPC bacteria showed relatively constant temperature preferences regardless of origin, whereas those of other coliform members gave varied results dependent upon their sources. The results provided circumstantial evidence that population densities of coliforms able to grow in EC broth at 43° and above but not in ordinary nutrient broth at 5° within 7 days of incubation in the environment are directly proportional to the degree of potential fecal contamination.

The coliform bacteria are well established as indicators of hazardous fecal contamination of water and food (1, 2). Apart from *Escherichia coli*, a member of this group, these bacteria occur frequently in areas apparently free from obvious fecal contamination, although all coliforms were once thought of as being of fecal origin (3). Thus, the sanitary significance of coliforms not related to fecal sources has been and still is of major concern, with much interest being directed

¹ Present address: Department of Biology, Faculty of Science, Tokyo Metropolitan University, Setagaya-ku, Tokyo 158, Japan.

toward their identity and potential pathogenicity.

There are a number of reports in the earlier literature that refer to an association of *Aerobacter* species with nonfecal materials such as soil (4–8) and plants (7, 9–12), although the genus of *Aerobacter* has been rejected and replaced with *Klebsiella* and *Enterobacter* (13–15). Also, the so-called intermediates have been reported to constitute the normal coliform flora of undisturbed soil and sites remote from potential polluting agents (7, 16–18). Usually, previous investigators classified their isolates on the basis of the results of four tests: indole, methyl red, Voges-Proskauer, and citrate, represented by the mnemonic IMViC. However, the taxonomic differentiation of coliforms by IMViC tests alone has remained ambiguous, especially between the genera *Klebsiella* and *Enterobacter* (19), both of which typically exhibit the --++ IMViC pattern. Besides, *Klebsiella* isolates are highly variable with respect to IMViC reactions (19–22). During the past decade, attempts have been made to identify isolates with further biochemical testing, such as with amino acid decarboxylases, and *Klebsiella pneumoniae* has increasingly been isolated from natural environments (20–24). This increase in published instances of the isolation of *K. pneumoniae* from the environment is partly because the taxonomic confusion with *Aerobacter* has been cleared up. Nevertheless, data on the species distribution of coliforms in areas not exposed to human and animal life are limited due to the former classification being on the basis of IMViC reactions.

Among a number of approaches used to estimate the sanitary significance of coliforms in the environment, the importance of temperature limits for growth, as well as that of the fecal coliform (FC) response (gas production at 44.5°), has been emphasized. BURMAN (25) reported that most coliforms of fecal origin, in contrast to those of nonfecal origin, grew at 42° and suggested that differentiation of coliforms by determining temperature preferences would provide more reliable evidence of fecal origin than IMViC typing. Several workers stated that the isolation of FC-positive (26, 27) or thermotolerant (ability to grow at 44.5°; 22) *Klebsiella* organisms from the environment would indicate their fecal/clinical origins. In addition, psychrotrophic coliforms characterized by their ability to multiply at 5° have been reported to constitute a significant proportion of coliforms in soil and stream water in areas remote from human life (28, 29). Chilled marine fish (30) and raw oysters (31) have been constant sources of such psychrotrophic coliforms as well.

The present study, therefore, was designed to compare the species composition and growth-temperature characteristics of coliforms in relation to their sources and to estimate the sanitary significance of each coliform member on the basis of their habitats and growth-temperature characteristics.

MATERIALS AND METHODS

Samples. Soil samples were collected from the mountainous area located around the Tokyo-Yamanashi prefectural border, where the soil was considered free from potential human contamination. These samples were taken by scraping away approximately 2 cm surface soil with sterile spoons and transferring into sterile glass bottles. All soil samples were either examined promptly upon return to the laboratory or were stored at 5° for periods of 15 to 20 hr before plating. Mountain stream water and polluted river water samples were collected from several locations on the Tamagawa River and its branches in Tokyo. Raw sewage samples were obtained at the Shibaura sewage treatment plant in Tokyo. The water samples were taken in sterile polyethylene bottles and stored in an insulated cooler from the time of collection until plating, usually not more than 6 hr. Feces samples were obtained from healthy individuals aged 20 to 63 and were plated within 24 hr.

Enumeration and isolation procedures. Desoxycholate agar (DA; Eiken) was used as the selective medium for enumerating coliforms. For plating, 10 g portions of soil samples were homogenized at high speed for 1 min in 90 ml of sterile, phosphate-buffered saline (1), and the resultant homogenates were allowed to settle for 15 min. Samples of 0.1 and 1 ml of the supernatant obtained were mixed with liquid DA tempered to 45 to 50° in standard petri dishes. In addition, 10 ml samples of the supernatant were filtered through Millipore membranes (HAWG047S0) with an average pore size of 0.45 μ m which were then placed on DA solidified in petri dishes. Coliforms in mountain stream water samples were enumerated by the membrane filter technique as above; 10 to 200 ml quantities of these samples were filtered through the membranes. Polluted river water and sewage samples were prepared for testing by serial dilution with sterile, phosphate-buffered saline, and 1 ml quantities of appropriate dilutions were plated with liquid DA. Feces samples were homogenized as above at a 1:100 dilution in sterile, phosphate-buffered saline. The resultant homogenates were diluted serially and plated using the agar-pore plate method. All inoculated plates were overlaid 15 min after plating with an additional 7 ml of the same medium. The DA overlay plates were made in duplicate for individual samples and incubated at 30 and 35° for 22 to 24 hr before counting. Typical red colonies recovered in the DA overlay incubated at 30 and 35° were presumptively recorded as 30°-total coliform (30-TC) and 35°-total coliform (35-TC) bacteria, respectively.

Presumed coliform colonies were subjected to the completed test based on the scheme of HORIE et al. (29). For individual samples, 10 to 30 colonies recovered in the DA overlay were picked randomly and inoculated into brilliant green bile lactose broth (Eiken) tubes. After incubation for 24 to 72 hr, the tubes positive for gas production were streaked on eosin methylene blue agar (Eiken) to ensure cultural purity. Well-isolated colonies on that medium were re-inoculated into lactose broth (polypepton (Daigo), 10 g; meat extract (Mikuni),

5 g; lactose, 5 g; 0.2% bromotymol blue solution, 15 ml; distilled water, 1,000 ml; pH 7.3) and then incubated for 24 to 72 hr, followed by confirmation of acid and gas production. Gas-producing cultures in lactose broth were transferred onto nutrient agar (Eiken) and tested for morphology, gram reaction (2), catalase (32), and cytochrome oxidase (33). Finally, gram-negative, rod-shaped, asporogenous isolates that were catalase-positive and oxidase-negative were recorded as completed coliforms (30- and 35-TCs). All culture media were incubated at 30°.

Biochemical testing for identification. Cultures grown in nutrient broth (Eiken) at 30° for 16 to 24 hr were used for biochemical tests. Unless otherwise indicated, all test media were incubated at 30° without shaking and final readings were taken after 6 days' incubation. IMViC reactions and hydrogen sulfide production were determined as described elsewhere (30). Motility was determined in the medium of BALL and SELLERS (34) incubated for 48 hr, and concurrently, gelatin liquefaction was recorded with this medium. The type of flagellation was determined by electron microscopy. Tests for lysine and ornithine decarboxylases and arginine dihydrolase were conducted using Møller's medium (Difco). The mode of glucose metabolism was determined in the oxidation-fermentation medium of HUGH and LEIFSON (35). Acid production from other carbohydrates was determined in the same medium containing each of the 1% filter-sterilized carbohydrates. Hydrolysis of alginate, pectate, Tween 80, and tributyrin was determined by the methods of DAVIS and EWING (36), PATON (37), SIERRA (38), and HUGO and BEVERIDGE (39), respectively. Hydrolysis of starch and deoxyribonucleic acid was recorded on nutrient agar containing 1% soluble starch and DNase test agar (Eiken), respectively. These tests for the hydrolytic activities were held for 10 days before being discarded as negative. Yellow pigment production was observed for cultures grown on nutrient agar, mannitol yeast extract agar (32), and nutrient skim milk agar (40) incubated at 25°. Tests for nitrate reduction in nitrate broth, phenylalanine deamination and malonate utilization in phenylalanine malonate medium, urea decomposition on Christensen's agar, gluconate oxidation, and growth in KCN broth were carried out by the methods described by SAKAZAKI (32). The isolates were classified by comparing the reactions obtained with those described by SAKAZAKI (15).

Determination of growth-temperature characteristics and FC response. Cultures grown in nutrient broth at 30° for 16 to 18 hr were inoculated into meat extract glucose (MG) broth (polypepton (Daigo), 10 g; meat extract (Mikuni), 3 g; sodium chloride, 3 g; glucose, 10 g; distilled water, 1,000 ml; pH 7.3) and into EC broth (Eiken). MG broth tubes were incubated in air-cooled incubators at 1 and 5° for 10 and 7 days, respectively, and in constant-temperature water baths at 37 and 40° for 24 hr. EC broth tubes were incubated in water baths at 43 and 44.5° for 24 hr. Temperature fluctuations in the air incubators and water baths were ± 0.5 and $\pm 0.1^\circ$, respectively, of each set temperature. Visible cultural

turbidity in the test tubes was taken as evidence of growth. The positive FC response of cultures was confirmed by their ability to grow and produce gas in EC broth at 44.5°.

RESULTS

Enumeration

Table 1 shows the results of the enumeration of coliforms in different samples. Relatively low coliform contents ($<10^3$ per g or 100 ml) were recorded for mountain soil and stream water samples, in which 30-TC counts were usually higher than 35-TC counts. Polluted river water, sewage, and human feces samples gave consistently high average coliform counts of the order of 10^5 , 10^7 , and 10^8 (per 100 ml or g), respectively, with no marked difference in the count between 30- and 35-TCs. Of the presumed coliforms recovered from the environmental samples, 19 to 53% were negatives for the completed test and were chiefly composed of anaerogenic *E. coli*, *Citrobacter* spp., and *Erwinia*-like bacteria (data not shown).

Biochemical reactions and identification

During the course of this study, 2,493 isolates were obtained from the five sources, and 2,372 (95%) thereof were divided into eight distinct groups, six species groups and two unidentified, on the basis of their biochemical reactions. Table 2 summarizes these results, with the data on the growth response at different temperatures of the isolates. All of the isolates were gram-negative, non-sporing, straight rods which produced catalase but not oxidase, fermented glucose in the O-F medium, formed acid and gas from lactose, and reduced nitrate to nitrite. Six (I-IVb) of the eight groups listed in the table were readily identified as being of the following species: *Escherichia coli*, *Citrobacter freundii*, *Citrobacter diversus*, *Klebsiella pneumoniae*, *Enterobacter cloacae*, and *Enterobacter aerogenes*. The isolates included in the remaining groups (V, VI) listed in the final two columns

Table 1. Enumeration of coliforms in different samples.

Sample type	No. of samples tested	Recovery (%) ^a		Log mean of presumed coliform counts per g or 100 ml		Accuracy (%) ^b	
		30-TC	35-TC	30-TC	35-TC	30-TC	35-TC
Mountain soil	36	94	83	2.34	2.04	47	58
Mountain stream water	37	99	96	2.53	1.76	74	68
Polluted river water	12	100	100	5.34	5.34	71	73
Raw sewage	8	100	100	7.11	7.18	80	81
Human feces	43	100	100	8.23	8.26	100	100

^a Percentage of samples from which recovered.

^b Percentage of colonies positive for the completed test.

Table 2. Characteristics of coliform isolates differentiated by biochemical tests.

Characteristic	% Isolates positive							
	Group I	IIa	IIb	III	IVa	IVb	V	VI
Indole production	97	5	100	27	1	0	0	0
Methyl red	100	100	100	15	2	0	0	100
Voges-Proskauer	0	0	0	98	100	100	100	0
Citrate (Simmons)	0	100	100	100	100	100	100	100
Motility	96	100	100	0	100	95	99	98
Pigmentation (nutrient agar)	0	0	0	0	0	0	1	9
H ₂ S production	1	94	0	0	0	0	0	0
Lysine decarboxylase	97	0	0	100	0	100	0	98
Ornithine decarboxylase	93	12	100	0	100	100	1	100
Arginine dihydrolase	26	34	25	1	100	0	72d	0
Phenylalanine deaminase	0	0	0	0	0	0	92w	0
Malonate utilization	0	2	100	100	73	100	100	100
Gelatin liquefaction	0	0	0	2	94d	14d	76d	0
Urease (Christensen)	ND	ND	ND	99	82	5	0	0
Tween 80 hydrolysis	ND	ND	ND	0	0	0	98d	98
Growth in MG broth at:								
1° (within 10 days)	0	0	0	0	0	0	100	100
5° (within 3 days)	0	5	0	0	8	5	100	100
5° (within 5 days)	0	83	64	23	72	24	100	100
5° (within 7 days)	0	97	76	44	81	24	100	100
37°	100	100	100	100	100	100	73	98
40°	100	100	97	99	98	100	0	0
Growth in EC broth at:								
43°	100	43	21	57	20	76	0	0
44.5°	100	3	15	47	12	48	0	0
FC response	93	1	9	19	5	19	0	0
No. of isolates								
30-TC	581	124	18	168	59	10	220	34
35-TC	559	222	15	239	88	11	12	12
Total	1140	346	33	407	147	21	232	46
Assumed species/group								
I <i>Escherichia coli</i>								
IIa <i>Citrobacter freundii</i>								
IIb <i>Citrobacter diversus</i>								
III <i>Klebsiella pneumoniae</i>								
IVa <i>Enterobacter cloacae</i>								
IVb <i>Enterobacter aerogenes</i>								
V Unidentified group (termed tentatively TPC group A)								
VI Unidentified group (termed tentatively TPC group B)								

Symbols: d, delayed reaction (after 3 or more days); w, weak reaction; ND, not done. For biochemical reactions for which all isolates were positive or negative, see text.

Table 3. Biochemical reactions of the TPC bacteria by additional tests.

Characteristic	% Positive for					
	140 isolates of TPC group A at:			36 isolates of TPC group B at:		
	48 hr	6 days	10 days	48 hr	6 days	10 days
Yellow pigment on:						
Nutrient agar	0	0	ND	11	11	ND
Mannitol yeast extract agar	6	7	ND	6	6	ND
Nutrient skim milk agar	76	ND	ND	72	ND	ND
Gluconate oxidation	64	ND	ND	100	ND	ND
Growth in KCN	77	77	ND	81	92	ND
Hydrolysis of:						
Starch	0	0	0	0	71	78
Alginate	0	0	0	0	0	0
Pectate	0	0	0	0	0	0
Tributyrin	0	0	83w	86	100	100
DNA	2	6	7	0	0	0
Acid from:						
L-Arabinose	100g	100	ND	100	100	ND
L-Rhamnose	100	100	ND	100	100	ND
L-Sorbose	0	0	ND	0	0	ND
Cellobiose	100	100	ND	6	9	ND
Raffinose	99	99	ND	53	57	ND
Sucrose	100	100	ND	0	0	ND
Melezitose	44	44	ND	0	0	ND
D-Adonitol	0	0	ND	100g	100	ND
Dulcitol	100g	100	ND	100g	100	ND
Glycerol	85	100	ND	92	100	ND
D-Mannitol	100g	100	ND	100	100	ND
D-Sorbitol	100g	100	ND	100	100	ND
Inositol	0	0	ND	100	100	ND
α -Methyl-D- glucoside	0	44w	ND	0	0	ND
Salicin	100g	100	ND	100	100	ND
Esculin	100	100	ND	78	92	ND

Symbols: w, weak reaction; g, more than 80% isolates positive for gas; ND, not done.

of the table gave such interesting results as suggested that they would not correspond in detail to any of the established species of the coliform group, although being homogenous in their biochemical reactions. This suggestion was supported by the results obtained in the examination of representative isolates of the two groups for further biochemical reactions (Table 3). Because all of the group V and VI isolates were able to grow at 5° within at least 3 days of incubation and even at 1° but not at 40° and above, they were tentatively termed "typical psy-

Fig. 1. Electron micrographs of peritrichously flagellated cells of TPC group A (A) and B (B). Scale-bar=1 μ m.

Cells grown on nutrient agar slants at 30° for 16 hr. Negatively stained with potassium phosphotungstate.

chrotrophic coliform (TPC)" bacteria and the former was designated TPC group A and the latter TPC group B in this study.

With no or few exceptions, the TPC group A isolates (IMViC — — + +) were motile by the use of peritrichous flagella (see Fig. 1-A), decarboxylated neither lysine nor ornithine, produced phenylalanine deaminase, utilized malonate as a sole carbon source, and hydrolyzed Tween 80 and tributyrin but not starch, alginate, pectate, or DNA. The reaction of phenylalanine deaminase was very weak, as the test medium turned faintly green 20 to 30 sec after the addition of the ferric chloride test reagent. Also, the lipolytic activity was barely detectable on the 10-day-old test medium and several isolates lost this activity upon sub-culture. Acid was produced from arabinose, rhamnose, cellobiose, raffinose, sucrose, dulcitol, glycerol, mannitol, sorbitol, salicin, and esculin. The acid production from arabinose, dulcitol, mannitol, sorbitol, and salicin was accompanied by gas bubble formation in most of the cultures. No acid was produced from sorbose, adonitol, or inositol. The majority produced a yellow, water-insoluble pigment on nutrient skim milk agar, with considerable variation of the degree of pigmentation, pale yellow being most common, but this property was not clear on nutrient agar or mannitol yeast extract agar. Although the TPC group A isolates were quite uniform as above, it was possible to separate them into

Table 4. Biogroups of the TPC group A isolates.

Characteristic	Biogroup:		
	1	2	3
Growth at 37°	+	+	—
KCN tolerance	+	—	v
Arginine dihydrolase	+	+	—
Gluconate oxidation	+	v	v
Acid from melezitose	+	—	—
Acid from α -methyl glucoside	(+)	—	—
No. of isolates tested	61	32	47

Symbols: +, more than 90% isolates positive; —, more than 90% isolates negative; v, 11–89% isolates positive; (+), delayed weak reaction.

three biogroups on the basis of growth at 37°, KCN tolerance, arginine dihydrolase, gluconate oxidation, and acid production from melezitose and α -methyl glucoside as shown in Table 4.

In general, the TPC group B isolates (IMViC — + — +) were motile by the use of peritrichous flagella (see Fig. 1-B), hydrogen sulfide-negative, lysine decarboxylase-positive, ornithine decarboxylase-positive, arginine dihydrolase-negative, phenylalanine deaminase-negative, and malonate-positive. Acid was produced from arabinose, rhamnose, adonitol, dulcitol, glycerol, mannitol, sorbitol, inositol, salicin, and esculin, whereas no acid was produced from sorbose, cellobiose, sucrose, melezitose, or α -methyl glucoside. Gas formation was observed simultaneously in most of the test tubes containing adonitol or dulcitol. The activities of Tween esterase and lipase were detected clearly within 48 hr of incubation. Alginate, pectate, or DNA was not hydrolyzed. No KCN inhibition was observed. The majority were yellow pigmented on nutrient skim milk agar.

Species composition in different samples

Table 5 shows the species/group composition of coliforms derived from the five sources. TPC group A was the most predominant type (>40%) of 30-TCs in mountain soil and stream water samples, but occurred in small amounts—less than 3% of the 35-TC isolates obtained from the same samples. About 20% of 30-TCs found in mountain soil and stream water samples, which might be also considered predominant, were represented by *C. freundii*, which was highest with about 40% of the 35-TC isolates derived from these sources. Also TPC group B was chiefly encountered in mountain soil and stream water samples. In contrast, *E. coli* and *K. pneumoniae* were major predominant types of both 30- and 35-TCs in polluted river water and sewage samples, comprising 66 to 92% of the isolates obtained from these samples. *E. coli* was detected consistently in the overwhelming majority from human feces samples. However, all other genera of the coliform group were found in these samples to some degree, and in two of

Table 5. Species composition of coliforms isolated from different sources.

Species/group	% Isolates											
	Mountain soil		Mountain stream water		Polluted river water		Raw sewage		Human feces			
	TC-30	TC-35	TC-30	TC-35	TC-30	TC-35	TC-30	TC-35	TC-30	TC-35		
<i>E. coli</i>	3.2	11.0	6.9	11.9	24.5	32.4	47.6	50.4	90.3	93.0		
<i>C. freundii</i>	20.0	39.0	20.2	40.1	7.9	2.0	4.8	—	1.0	—		
<i>C. diversus</i>	3.2	3.0	2.5	1.4	—	2.0	1.6	—	0.4	0.5		
<i>K. pneumoniae</i>	10.3	24.5	7.6	18.4	41.1	52.9	32.5	41.5	4.4	4.7		
<i>E. cloacae</i>	9.2	14.0	6.3	13.8	9.3	3.9	6.3	2.4	—	0.9		
<i>E. aerogenes</i>	0.5	—	—	1.1	—	2.9	—	3.3	1.8	—		
TPC group A	44.9	3.0	40.7	1.7	5.3	—	—	—	—	—		
TPC group B	5.4	4.5	6.3	0.8	2.6	—	—	—	—	—		
Not determined	3.2	1.0	9.4	10.7	9.3	3.9	7.1	2.4	2.2	0.9		
No. of isolates	185	200	317	354	151	102	126	123	505	430		

the 43 samples tested *K. pneumoniae* occurred alone (data not shown). In addition, sewage and feces samples were devoid of the TPC bacteria.

Growth-temperature characteristics of coliforms in relation to their sources

In testing the growth response at different temperatures, the isolates of *E. coli* and the TPC bacteria showed relatively constant features; namely, all of the former grew at 44.5° but not at 5° while all of the latter grew well at 5° with a failure to grow at 40° and above (Table 2). Also, positive FC reactions were produced by most (93%) of the *E. coli* isolates. These results indicate that the growth-temperature characteristics of these coliform types are independent of their origins. On the other hand, the isolates of the other members gave variable results for the growth response at 5, 43, and 44.5° and for the FC test. Attention therefore was directed to the relationships between these characteristics of the isolates identified as being of the species of *Citrobacter*, *Klebsiella*, and *Enterobacter* and their sources.

As shown in Fig. 2, the greater majority of the *Klebsiella* and *Enterobacter*

Fig. 2. Growth response at 5, 43, and 44.5° of some members of coliforms in relation to their sources.

isolates obtained from mountain soil and stream water samples were psychrotrophic types that grew in MG broth at 5° but not in EC broth at 43 and 44.5°, whereas thermotolerant types capable of growth at such high temperatures constituted considerable proportions of those derived from polluted river water and sewage samples, with a marked reduction of positives at 5°. Apparently, the occurrence of FC-positive isolates was directly proportional to that of the thermotolerant types. These tendencies were also exhibited by the *Citrobacter* isolates but were not so pronounced as those shown by the isolates of the former genera. With the exception of the *Citrobacter* isolates, all of fecal origin grew at 43° but not at 5°.

Consequently, few psychrotrophic coliforms occurred among the isolates able to grow at 43° and above during the course of this study.

DISCUSSION

The present study has demonstrated the existence of two unidentified groups of psychrotrophic coliforms, designated tentatively as TPC groups A and B, respectively. In general, both the TPC group A and B isolates grew at 1° but not at 40°, produced a non-diffusible yellow pigment on nutrient skim milk agar, fermented dulcitol with gas production, and showed the activities of Tween esterase and lipase, besides the former being weakly positive for phenylalanine deaminase (Tables 2 and 3). These results provide interesting information on the taxonomic positions of the TPC bacteria, since these properties are limited to members of only few groups of the family *Enterobacteriaceae*. It has been reported that psychrotrophic coliforms usually give the — — + + IMViC pattern (28–31, 41–43), strains resembling *Serratia liquefaciens*, formerly designated as *Aerobacter liquefaciens* or *Enterobacter liquefaciens*, being most common (42, 43). Lipolytic activity has been chiefly associated with *Serratia* and *Proteus* species within *Enterobacteriaceae* (15, 36), the latter being entirely out with the coliform concept. These facts would suggest that TPC group A has some degree of phenotypic similarity to *S. liquefaciens*, but this relationship is discounted on comparing other basic characteristics of the two. Yellow-pigmented bacteria are common among strains belonging to the *Erwinia* species included in the herbicola group (40, 44–46), one of the three clusters within this genus, for which EWING and FIFE (47) proposed the name *Enterobacter agglomerans*. Also, the organism previously referred to as “yellow-pigmented *Enterobacter cloacae*” has been placed in a new species *Enterobacter sakazakii* (48). Interestingly, some strains of the two yellow-pigmented *Enterobacter* species produce phenylalanine deaminase (47, 48), suggesting some resemblance between TPC group A and these species. However, our data indicate that TPC group A differs apparently from *E. sakazakii* in other basic characteristics, such as ornithine decarboxylase. In view of the experimental evidence so far obtained, the outline of TPC group A as a taxon is

considered clear, this group seeming to resemble *E. agglomerans* most closely, although *E. agglomerans* or the herbicola group is a heterogenous group with significant biochemical diversity (15, 49, 50). TPC group B, which is also considered to be a distinct group, corresponds to *C. freundii* with respect to IMViC reactions, but the present results would suggest that this group is similar to *Enterobacter* or *Erwinia* species rather than *C. freundii*. At any rate, further studies are necessary to pinpoint the exact taxonomic positions of the TPC bacteria.

There is general agreement that the — — + + and — + — + IMViC types constitute a significant proportion of coliforms in areas free from fecal contamination. Analysis of the species distribution as given in this paper (Table 5) indicates that TPC group A and *C. freundii* are major predominant coliforms in unpolluted areas remote from human life. Likewise, there is no doubt that TPC group B originates in such areas. Due to few attempts having been made to identify isolates beyond IMViC reactions in the past, it is difficult to interpret previous reports as to the relationships of the reported predominant types in the environment to the TPC bacteria and *C. freundii*, despite the fair agreement existing between their results and ours concerning the occurrence of IMViC types. However, most of the psychrotrophic coliforms that have been reported by HORIE et al. (28, 29) to be dominant in mountainous areas are considered identical with the TPC bacteria, in the light of their biochemical properties and habitats.

The data presented here raise the question of whether the TPC bacteria and *C. freundii* predominating in unpolluted areas are established in the soil and stream water in these areas. As stated by RANDALL (17), it is difficult to conclude that the coliform types are established in these environments, because of the low population densities thereof that were not of the order expected of bacteria indigenous to such environments. However, it would appear evident that the sources of the coliforms lay elsewhere than in warm-blooded animal feces. For example, *E. agglomerans* or the herbicola bacteria, probably related to TPC group A, are associated with plants as constituents of the saprophytic flora (46, 47), and *C. freundii* or the — + — + IMViC type has been found as the predominant coliform in the intestinal tract of cold-blooded animals such as freshwater fish (51), aquarium snails (52), and frogs (53).

The present results (Table 5) also indicate that although ubiquitous in nature, *K. pneumoniae* is rather a predominant coliform in polluted areas apparently associated with fecal contamination. In addition, this organism was second to *E. coli* in frequency of occurrence in human feces, in some cases occurring alone. These findings support to some extent the view of BUTTIAUX and MOSSEL (54) that *Klebsiella* organisms, as well as *E. coli*, are specific indicators of fecal pollution.

The sanitary significance of *E. coli*, as generally accepted, is unquestionable. Also, the results obtained with the distribution of the TPC bacteria lead us to

conclude that they would be of no value as fecal indicators. With respect to the sanitary significance of other coliform members, their growth-temperature characteristics may be useful in the estimation thereof. In this study, with few exceptions of *Citrobacter* isolates, all coliforms of fecal origin were able to grow in EC broth at 43° but not in MG broth at 5° within 7 days of incubation, and the incidence of such coliforms with the high temperature preference was evidently higher in polluted river water and sewage samples than in mountain soil and stream water samples (Fig. 2). These data provide circumstantial evidence that population densities of coliforms able to grow in EC broth at 43° and above but not in ordinary nutrient broth at 5° within 7 days of incubation in the environment are directly proportional to the degree of potential fecal contamination. In this context, BAGLEY and SEIDLER (26) concluded that there were insufficient data to equate the mere ability of a coliform to grow in EC broth at 44.5° with its fecal origin. Nevertheless, we prefer to consider that the sanitary significance of coliforms with the high temperature preference should not be discounted, even if FC negative.

One should note that lowering the incubation temperature to 30° for enumerating coliforms resulted in higher counts than were obtained at 35° when examining samples taken from unpolluted areas (Table 1). This is probably attributable to the presence of a large number of the TPC bacteria in such areas. To eliminate these coliform types, therefore, presumptive tests for total coliforms should be carried out with incubation at 35° and above. Furthermore, if these are made at such elevated temperatures as 43°, not only *E. coli* but all coliform members of probable fecal/clinical origins may be recovered from the environment, simultaneously with the elimination of coliforms which are of limited sanitary significance.

We are grateful to K. Saheki, Department of Food Science and Technology, Tokyo University of Fisheries, for the interest shown and encouragement given during this work. We also thank T. Yasuda and T. Kobayashi for their technical assistance. Special thanks are made to T. Maruyama, Department of Biology, Tokyo Metropolitan University, for electron micrographs.

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