

## Distribution of Ribonucleic Acid Coliphages in Raw Sewage from Treatment Plants in Japan

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To determine the transmission cycle of ribonucleic acid (RNA) coliphages in their natural habitats, we investigated the distribution patterns of RNA phages in raw sewage collected from treatment plants in various localities in Japan. Most of the sewage samples contained group II and III phages. Samples from treatment plants in Sapporo, Tokyo, and Toyama contained appreciable amounts of group I phages in addition to the group II and III phages. As a whole, raw sewage from treatment plants in Japan contained RNA phages of the three groups in the ratio 1:2:5, group I/II/III. Based on the distribution patterns of RNA phages in sewage from domestic drainage in Japan proper (group II/III, 3:1), in animal feces and sewage from slaughter houses (mostly group I), and in human feces (group II/III, 1:1), it can be reasonably said that group I phages tend to be introduced from animal sources and group II and III phages tend to be introduced from human sources. Raw sewage from treatment plants in Japan consists mainly of human feces, sewage from domestic drainage, and industrial wastewater, and, in part, from slaughter houses. In fact, sewage from slaughter houses together with that from human sources flowed into the treatment plants of Tokyo as far as we could confirm.

Ribonucleic acid (RNA) coliphages have been classified into four groups (I through IV) according to their serological and physicochemical properties (7, 11).

In the course of our systematic surveys on RNA phages, we chose sewage samples from domestic drainage and feces of certain domestic animals and of humans as sources for the isolation of such phages. Analysis of these three types of material revealed a unique distribution pattern of RNA phages in each material examined. That is, the sewage from domestic drainage in Japan proper contained group II and III phages in a 3:1 ratio (4, 6); the sewage from slaughter houses, feces of domestic animals, and feces of animals in zoological gardens contained group I phages exclusively; and the feces of humans contained group II and III phages almost equally, although the frequencies of isolation of these phages in human and animal feces were fairly low (2 and 6%, respectively) as far as we could determine (9). Thus, in general, group I phages were considered to be of animal origin, and group II and III phages were thought to be of human origin.

In this connection, it was of interest to investigate the distribution patterns of RNA phages in raw sewage from treatment plants. Raw sewage

from treatment plants consists mainly of human feces, sewage from domestic drainage, and industrial wastewater, and, in part, of sewage from slaughter houses, and would be expected to contain mostly group II and III phages, with a minor fraction of group I phages.

### MATERIALS AND METHODS

**Media.** Peptone-glucose medium (5 g of NaCl, 20 g of peptone, 2 g of glucose, 1,000 ml of water, pH 7.4) was used for collection of sewage samples, isolation of RNA phages, and dilution of phage and antiphage sera; peptone-glucose medium supplemented with 0.25% yeast extract and 0.01 M CaCl<sub>2</sub> was used for the preparation of crude phage lysate.

**Bacterial strains and antiphage sera.** Bacterial strains (*Escherichia coli* K-12 strains  $\lambda$ 1 [F<sup>+</sup>], Q13 [Hfr], and W3110 [F<sup>-</sup>]) and antiphage sera against phages MS2 (group I), GA and JP34 (II), Q $\beta$  and VK (III), and SP (IV) were the same as those described previously (1, 6, 8).

**Collection of samples and preparation of original phage samples.** Approximately 10 ml of raw sewage was collected in small sterilized plastic tubes from each of the treatment plants listed in Table 1 and brought back to our laboratory under normal temperature conditions within 2 days, except for the samples from Recife (Brazil), New York (N.Y.), and Würzburg (West Germany). Two milliliters of the raw sewage was mixed with the same volume of peptone-

glucose medium, treated with 0.5 ml of chloroform to kill bacteria, and subjected to low-speed centrifugation (Sorvall GLC 1) to remove bacterial debris and certain other precipitates. The supernatant fractions so obtained were stored in a cold room (4°C) and used as original phage samples.

**Isolation and grouping of RNA phages by the serological method.** The isolation of RNA phages was performed as described previously (8). Each 0.1 ml of original phage sample was plated on *E. coli* strains A/λ (F<sup>+</sup>), Q13 (Hfr), and W3110 (F<sup>-</sup>), and single plaques on the individual plates were picked up, suspended, and stirred vigorously in 2 ml of peptone-glucose medium with 0.5 ml of chloroform. The samples were left standing for several minutes, after which the supernatant fraction was used for further analysis. In the present study, we picked up 30 to 70 plaques per sample. These phage stocks were subjected to spot tests on *E. coli* strains A/λ, Q13, W3110, and A/λ with ribonuclease (100 μg/plate). Phages which lysed strain A/λ or Q13, or both, but did not lyse strains W3110 and A/λ with ribonuclease were picked up, purified several times by the single-plaque isolation method, and stocked in a cold room (4°C) as RNA phages. RNA phages infect specifically male host strains and are sensitive to ribonuclease treatment.

Grouping of RNA phages by the serological method was carried out as described previously (6, 10). In short, newly isolated RNA phages were subjected to spot tests on strain A/λ or Q13 with ( $K = 1$  per plate) or without antiserum of six standard phages (MS2 [group I], JP34 and GA [II], Qβ and VK [III], and SP [IV]).

## RESULTS

**Density of total coliphages in samples.** The densities of total coliphages were fairly high, ranging from 10 to 10<sup>6</sup> plaque-forming units per ml of original phage sample (with many showing 10<sup>2</sup> to 10<sup>4</sup> plaque-forming units per ml), in almost all of the samples collected from treatment plants in various localities in Japan (Table 1). Although we observed a fluctuation in density of the order of 10<sup>2</sup> in surveys of raw sewage at 2-h intervals during the day (data not shown), we usually collected sewage samples from treatment plants in the afternoon. There appears to be a tendency toward somewhat higher phage densities in the summer (10<sup>4</sup> to 10<sup>5</sup> plaque-forming units per ml) than in the winter (10 to 10<sup>3</sup> plaque-forming units per ml).

**Density of RNA phages in samples.** To obtain as many RNA phages as possible from the raw sewage of treatment plants, we picked up plaques from only A/λ (F<sup>+</sup>) and Q13 (Hfr) plates (not from W3110 [F<sup>-</sup>] plates) and rejected plaques which showed an apparently deoxyribonucleic acid phage morphology. As a result, the number of RNA phage strains per total number of phages tested does not give the proportion of RNA phages in a given sample. The density of RNA phages in a given sample was

determined by subtracting the number of plaque-forming units on ribonuclease containing plates (100 μg/plate) from the total plaque-forming units on ribonuclease-free plates.

Almost all of the samples contained RNA phages at relatively high densities, i.e., 5 to 90% of the total coliphages (with many at 30 to 60%). Thus, RNA phages constituted the predominant phage species in sewage from treatment plants, as in the case of sewage from domestic drainage (2, 3, 6, 8).

**Distribution pattern of RNA phages in collected materials.** We picked up 3,243 plaques and obtained 1,832 RNA phage strains (Table 1). By serological analysis, these were classified into four groups, (I/II/III/IV, 221:487:1,122:2). Most of the sewage samples contained only group II and III phages. Samples from the treatment plants in Sapporo, Tokyo, and Toyama contained appreciable amounts of group I phages in addition to the group II and III phages. As a whole, raw sewage from treatment plants in Japan contained RNA phages of the three groups in the ratio 1:2:5, group I/II/III. Excluding the data for group I, group II and III phages were isolated at a ratio of 1:1 in Hokkaido and the Tohoku district, but at a ratio of 1:3 south of the Kanto district. Thus, the two prominent features in the distribution patterns of RNA phages in raw sewage from treatment plants in Japan were the predominance of group III phages over group II phages and the existence of group I phages in certain restricted treatment plants (Sapporo, Tokyo, and Toyama).

Continuous surveys over 3- to 5-year periods of the distribution patterns of RNA phages in raw sewage from the Shinkawa (Sapporo), Shibaura (Tokyo), Jonan (Takasaki), and Chubu (Fukuoka) treatment plants (see Table 1) have revealed a continuity of RNA phages in the sample materials, although the presence of RNA phages fluctuated more widely than that in domestic drainage (4).

In addition, raw sewage and sludge collected from treatment plants also contained many RNA phages, and the group types of RNA phages isolated were roughly the same in both materials (data not shown).

## DISCUSSION

The most prominent features in the distribution patterns of RNA phages in raw sewage collected from treatment plants in Japan are the predominance of group III phages over group II phages and the existence of appreciable amounts of group I phages as well as group II and III phages, in contrast to the sewage from domestic drainage. Based on the distribution patterns of RNA phages in sewage from domestic drainage

TABLE 1. Density of total coliphages and the distribution pattern of RNA phages in raw sewage collected from treatment plants of various localities in Japan

District	City	Treatment plant	Density of total coliphages <sup>a</sup>	No. of phages tested	No. of RNA phage strains	RNA phage group (no.)				Date of collection	
						I	II	III	IV		
Hokkaido	Sapporo	Soseigawa	$1.3 \times 10^3$	70	34	2	30	2	0	27 Sept. 1973	
	Sapporo	Soseigawa	$5.6 \times 10^3$	60	44	0	0	44	0	28 Aug. 1974	
	Sapporo	Shinkawa	$3.0 \times 10^4$	74	68	0	68	0	0	27 Sept. 1973	
	Sapporo	Shinkawa	$3.9 \times 10^3$	75	57	30	0	27	0	28 Aug. 1974	
	Sapporo	Shinkawa	$6.3 \times 10^4$	101	96	17	43	36	0	8 Oct. 1975	
	Sapporo	Shinkawa	$4.3 \times 10^4$	65	56	0	6	50	0	5 Sept. 1976	
	Total				445	355	49	147	159	0	
Tohoku	Hachinohe	Asahigaoka	$2.4 \times 10^4$	45	26	5	21	0	0	24 Nov. 1975	
	Hachinohe	Korekawa	$1.5 \times 10^4$	49	30	0	24	6	0	24 Nov. 1975	
	Sendai	Minamigamou	$2.0 \times 10^3$	92	48	0	2	46	0	11 Aug. 1973	
	Sendai	Minamigamou	$2.8 \times 10^4$	67	3	0	0	3	0	10 Sept. 1974	
	Total				253	107	5	47	55	0	
Kanto	Maebashi	Maebashi	$4.0 \times 10^4$	71	34	0	4	30	0	29 Aug. 1973	
	Takasaki	Jonan	$4.0 \times 10^4$	50	41	0	8	33	0	30 Aug. 1973	
	Takasaki	Jonan	$4.0 \times 10^4$	64	30	0	6	24	0	30 Aug. 1973	
	Takasaki	Jonan	$3.2 \times 10^5$	77	14	0	0	14	0	28 July 1974	
	Takasaki	Jonan	$1.9 \times 10^3$	56	49	4	32	13	0	21 Dec. 1975	
	Tokyo	Ochiai	$2.0 \times 10^3$	104	53	0	4	49	0	19 Sept. 1973	
	Tokyo	Ochiai	$6.3 \times 10^2$	71	40	21	7	12	0	21 Dec. 1973	
	Tokyo	Ochiai	$1.0 \times 10^3$	40	25	18	4	3	0	22 Mar. 1974	
	Tokyo	Shibaura	$2.0 \times 10^3$	78	52	6	24	22	0	19 Sept. 1973	
	Tokyo	Shibaura	$7.0 \times 10^2$	40	31	0	0	30	1	21 Dec. 1973	
	Tokyo	Shibaura	$7.0 \times 10^2$	50	34	21	4	9	0	22 Mar. 1974	
	Tokyo	Shibaura	$4.5 \times 10^2$	98	66	30	3	32	1	27 Dec. 1974	
	Tokyo	Shibaura	$8.0 \times 10^2$	169	92	40	13	39	0	5 Apr. 1975	
	Tokyo	Shibaura	$1.0 \times 10^4$	80	46	2	39	5	0	8 July 1975	
	Tokyo	Shibaura	$5.0 \times 10^4$	108	104	0	17	87	0	21 June 1976	
	Tokyo	Shibaura	$2.0 \times 10^3$	70	49	5	6	38	0	5 Oct. 1977	
	Tokyo	Ogasawara	$2.0 \times 10^2$	16	15	0	0	15	0	5 Nov. 1973	
	Choshi	Choshi	$2.0 \times 10^5$	44	0	0	0	0	0	1 Sept. 1973	
	Total				1,286	775	147	171	455	2	
	Chubu	Niigata	Funami	$1.0 \times 10^4$	50	41	0	6	35	0	6 Sept. 1973
Niigata		Funami	$2.9 \times 10^3$	82	44	0	0	44	0	14 Sept. 1974	
Niigata		Funami	$2.0 \times 10^3$	26	8	0	8	0	0	28 Dec. 1975	
Toyama		Ushijima	$8.0 \times 10^4$	62	4	1	2	1	0	6 Oct. 1973	
Toyama		Ushijima	$8.0 \times 10^2$	39	31	14	6	11	0	21 Mar. 1974	
Toyama		Ushijima	$7.2 \times 10^3$	70	14	0	0	14	0	15 Sept. 1974	
Toyama		Ushijima	$1.4 \times 10^2$	43	21	2	12	7	0	29 Dec. 1975	
Total					372	163	17	34	112	0	
Kinki	Osaka	Ebie	$7.0 \times 10^3$	73	47	0	0	47	0	5 Oct. 1973	
	Osaka	Nakahama	$5.0 \times 10^3$	73	69	1	23	45	0	5 Oct. 1973	
	Total				146	116	1	23	92	0	
Chugoku	Hiroshima	Eba	$1.5 \times 10^6$	42	16	0	6	10	0	30 July 1973	
	Total				42	16	0	6	10	0	
Kyushu	Fukuoka	Chubu	$1.4 \times 10^3$	45	2	0	1	1	0	1 Nov. 1973	
	Fukuoka	Chubu	$2.6 \times 10^6$	62	43	0	0	43	0	6 Aug. 1974	
	Fukuoka	Chubu	$2.2 \times 10^2$	27	0	0	0	0	0	3 Jan. 1976	
	Fukuoka	Chubu	$4.2 \times 10^2$	47	38	2	10	26	0	11 Apr. 1976	
	Fukuoka	Chubu	$1.9 \times 10^6$	67	65	0	0	65	0	10 Aug. 1976	
	Kurume	Kurume	$3.0 \times 10^2$	72	17	0	15	2	0	2 Nov. 1973	
	Kurume	Kurume	$6.0 \times 10^2$	67	30	0	10	20	0	28 Nov. 1973	
	Kumamoto	Kumamoto	$1.5 \times 10^6$	35	11	0	0	11	0	12 Aug. 1976	
	Kumamoto	Kusunoki-danchi	$4.0 \times 10^6$	30	0	0	0	0	0	12 Aug. 1976	
	Kagoshima	Kinko	$6.0 \times 10^3$	67	31	0	23	8	0	19 July 1973	
	Kagoshima	Kinko	$1.5 \times 10^4$	110	7	0	0	7	0	4 Aug. 1975	
	Total				629	244	2	59	183	0	
	Okinawa	Naha	Naha	$2.7 \times 10^3$	70	56	0	0	56	0	13 Sept. 1974
Total					70	56	0	0	56	0	
Grand total					3,243	1,832	221	487	1,122	2	

<sup>a</sup> Plaque-forming units per milliliter of original phage sample.

in Japan proper (group II/III, 3:1) (4, 6), in animal feces and sewage from slaughter houses (mostly group I), and in human feces (group II/III, 1:1) (9), it can be reasonably said that the group I phages observed in raw sewage from treatment plants tend to be introduced from animal sources (feces of domestic animals and sewage from slaughter houses) and group II and III phages tend to be introduced from human sources (human feces and sewage from domestic drainage). Raw sewage from treatment plants consist mainly of human feces, sewage from domestic drainage, industrial wastewater, and in part, sewage from slaughter houses. In fact, sewage from slaughter houses together with that from human sources flowed into the treatment plants of Tokyo as far as we could confirm.

Group I RNA phages amplified in the alimentary tract of animals (and also presumably in sewage from slaughter houses) probably reinfect and propagate again in the alimentary tract of animals through food and water contaminated with RNA phages excreted from animals in the external environment. Thus, the transmission cycle of group I phages should be complete. The same situation can be expected to occur between humans and the group II and III phages, although the frequencies of isolation of these phages in human feces were fairly low as far as we could determine. Consequently, sewage from

treatment plants in Japan contained mostly group III and II phages, with a minor fraction of group I phages, as might be expected from the distribution patterns of RNA phages in the human and animal feces (9) and in sewage from domestic drainage (4, 6). The predominance of group III phages over group II phages in raw sewage from treatment plants in Japan appears to suggest a wider contribution from human feces than from sewage from domestic drainage and a selective amplification of group III phages in the raw sewage.

The distribution patterns of RNA phages in raw sewage collected from treatment plants in several countries are summarized in Table 2. Sewage samples from Recife (Brazil) and Würzburg (West Germany) contained only group I phages, although we were unable to ascertain whether or not the samples included sewage from slaughter houses. Most of the sewage samples contained RNA phages of group II or III, or both, at fairly high densities, comparable to those in the treatment plants of Japan.

In contrast to the preferential relationships observed between RNA phage groups and animal species (group II and III phages with humans, and group I phages with other animals) (9), there appear to be no special phage-host relationships between RNA phage groups and human races. It can be said that (i) the most

TABLE 2. Distribution patterns of RNA phages in raw sewage collected from treatment plants in various countries

Country	City	Density of total coli-phages <sup>a</sup>	No. of phages tested	No. of RNA phage strains	RNA phage group (no.)				Date of collection	Reference
					I	II	III	IV		
Mexico	Campeche	$5 \times 10^2$	8	0	0	0	0	0	3 Apr. 1973	5
Saudi Arabia	Kahfuji	$1 \times 10^3$	43	0	0	0	0	0	March 1973	5
Australia	Sidney	$2 \times 10^4$	70	10	0	10	0	0	19 Aug. 1973	5
United States	Oakland (Calif.)	$10^2$ - $10^8$	198	154	0	52	102	0	29 Aug. 1973	5
	Oakland (Calif.)	$3 \times 10^2$	50	28	1	27	0	0	7 Dec. 1973	5
	Richmond (Calif.)	$5 \times 10^4$	52	51	0	0	51	0	7 Dec. 1973	5
	New York (N.Y.)	$2 \times 10^2$	48	45	0	45	0	0	Jan. 1976	Unpublished data
	San Elijo (Calif.)	$5 \times 10^3$	28	7	0	1	6	0	10 Aug. 1977	Unpublished data
Brazil	Recife	$3 \times 10^2$	22	4	4	0	0	0	27 Nov. 1975	Unpublished data
The Philip-pines	Manila	$1 \times 10^3$	33	3	0	0	3	0	3 Nov. 1976	8
West Germany	Würzburg	$5 \times 10^3$	58	17	17	0	0	0	June 1978	Unpublished data
	Würzburg (University Hospital)	$5 \times 10^4$	51	6	0	6	0	0	June 1978	Unpublished data

<sup>a</sup> Plaque-forming units per milliliter of original phage sample.

predominant RNA phage species in raw sewage from treatment plants are those of groups III and II, both in Japan and in several other countries (Table 2); (ii) raw sewage from treatment plants is thought to consist mainly of wastewater of human origin, including human feces, sewage from domestic drainage, and industrial wastewater, both in Japan and in the several other countries listed in Table 2; and (iii) group II and III phages are thought to be primarily of human origin, based on direct analysis of fecal samples (9).

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