

## Isolation of *Yersinia ruckeri* Bacteriophages

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Eight bacteriophages effective against *Yersinia ruckeri*, the enteric redmouth disease bacterium, were isolated. Phage YerA41, a tailed icosahedral virus isolated from sewage enrichments, lysed 34 of 35 strains of *Y. ruckeri* serovar I, but was inactive against 15 strains belonging to three other serological groups. Six other phages lysed strains of serovars II, V, and I', a subgroup of serovar I. YerL62, a phage obtained by mitomycin C induction, was specific for one of three serovar V strains. These bacteriophages, particularly YerA41, have potential value for fish disease diagnostic work.

*Yersinia ruckeri* is the causative agent of enteric redmouth disease of salmonid fish (3, 6). As this disease can cause severe economic losses in aquaculture and hatchery operations, fish health protection programs monitor fish stocks for the presence of the bacterium. Recently, concern about the enteric redmouth bacterium has increased, as it has been found associated with disease in fish in the eastern United States (2), France (10), and the Federal Republic of Germany (8), and with apparently healthy fish tested in routine monitoring (15). Previously, both the organism and the disease were thought to be confined to the western part of North America and spread only by the movement of infected fish (2).

Diagnostic laboratories use a sequence of selective media, biochemical tests, and serology to detect and identify *Y. ruckeri* (13), but none of these procedures used alone will give an unambiguous identification, and, in combination, their interpretation requires considerable time and technical skill. The problems of diagnosis have become more complex with the isolation of several serological varieties of *Y. ruckeri*, in addition to the first-isolated Hagerman serotype (serovar I) (14, 14a). These varieties may have different degrees of virulence for salmonid species (2, 12), but as yet, this is not resolved.

Bacterial sensitivity to bacteriophages provides the possibility of using phage typing as an epidemiological tool and as an aid to rapid diagnosis. Previously, we had been unable to isolate bacteriophages effective against *Y. ruckeri* (15). Here we report on phages which we subsequently isolated and on some differences in their host specificities that have potential diagnostic applications.

### MATERIALS AND METHODS

**Bacterial strains.** The *Y. ruckeri* strains used and their isolation sources are listed in Table 1. Detailed descriptions of strains RS1 through RS46 have been given previously (4, 15). For additional cultures included in this study, we are grateful to J. Warren and D. Desens, U.S. Fish and Wildlife Service, Fish Disease Control Center, La Crosse, Wisc.; T. M. Cook, Department of Microbiology, University of Maryland, College Park, Md.; K. Johnson, Fish Vaccine

Unit, Connaught Research Institute, Toronto, Ontario, Canada; J. G. Daly, Fish Health Laboratory, Department of Microbiology, University of Guelph, Ontario; B. Lindvik, British Columbia Fish and Wildlife, Nanaimo, British Columbia, Canada; and K. Böhm, Institut für Mikrobiologie und Tierseuchen der Tigrärztlichen Hochschule Hannover, Hanover, Federal Republic of Germany.

*Yersinia enterocolitica* strains used were four isolates from humans, provided by S. Toma, Ontario Ministry of Health, Toronto, Canada; a fish isolate (ONT367) from J. G. Daly; and strains 2635, 2383, and WA, kindly provided by P. Gemski, Walter Reed Army Institute of Research, Washington, D.C. All other bacterial strains were from the culture collection of the Department of Microbiology, University of Guelph. Working cultures were maintained on slants of Trypticase soy agar (BBL Microbiology Systems) at 15°C and grown in Trypticase soy broth (BBL Microbiology Systems) at 25°C. Strains were identified with biochemical and serological tests as described previously (14a, 15).

**Phage isolation and titration.** In all phage titrations and tests, phage top agar was used (8 g of tryptone, 5 g of NaCl, and 7.5 g of agar per liter), and Trypticase soy agar plates were supplemented with MgCl<sub>2</sub> and CaCl<sub>2</sub> to a final concentration of 1 mM. We followed the procedures given by Baker and Farmer (1) for isolation of bacteriophages from sewage enrichments. Sewage samples were collected from the Water Pollution Control Plant, Guelph, Ontario, and enrichments were prepared with the first 44 cultures listed in Table 1. All enrichments and phage titrations were carried out at 25°C. Three successive plaque purifications were carried out on each phage isolate.

Phage stocks were prepared by soaking plates showing near-confluent lysis with Trypticase soy broth for 2 to 4 h. The liquid was collected, centrifuged at low speed to remove cells, and passed through a 0.45- $\mu$ m pore-size membrane filter. Phage stocks were stored at 4°C and titrated at 10-day intervals to assess stability.

**Lysogenic induction.** Mitomycin C (Sigma Chemical Co.) was added at a final concentration of 1  $\mu$ g/ml to 2-h-old Trypticase soy broth cultures of *Y. ruckeri* grown at 25°C with shaking. After 18 h of growth, the cells were removed by centrifugation, and 10 ml of the supernatant fluid was vigorously mixed with 1 ml of chloroform and left at 40°C for 1 h. Samples from the aqueous layer were placed in plastic multiwell trays and aerated in a laminar flow for 30 min. These samples were spotted onto lawns of bacteria which were later examined for plaques.

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TABLE 1. *Yersinia ruckeri* strains used in this study

Laboratory Code	Other designation	Source <sup>a</sup>	Serovar	Laboratory Code	Other designation	Source <sup>a</sup>	Serovar
RS1	ONT270	Brown trout, Ontario	IV	RS38	JW77-46	Rainbow trout, Indiana	I
RS2	ONT258	Rainbow trout, Ontario	II	RS39	JW77-71	Rainbow trout, Ohio	I
RS3	BC74	Chinook salmon, Oregon	II	RS40	JW78-74	Rainbow trout, Wisconsin	I
RS4	FHL 11.4	Rainbow trout, Utah	I	RS41	JW79-52	Rainbow trout, Wisconsin	I
RS5	ERM II	Unknown	I	RS42	JW81-29	Rainbow trout, Missouri	I
RS6	ONT193	Brook trout, Ontario	II	RS43	JW81-48-4	Rainbow trout, Ohio	I
RS7	RO8-85	Muskrat, Ontario	I	RS44	82-091	Rainbow trout, British Columbia	II
RS8	ONT288	Cisco, Ontario	II	RS45	82-077	Steelhead trout, British Columbia	I
RS9	9-4-5	Rainbow trout, Nova Scotia	I	RS46	JW82A	Rainbow trout, Ohio	I
RS10	ONT339	Brown trout, Ontario	II	RS47	JW82B	Rainbow trout, Ohio	I
RS11	ATCC 29473 <sup>b</sup>	Rainbow trout, Idaho	I	RS48	OS76	Rainbow trout, Oregon	I
RS12	ATCC 29908	Rainbow trout, Washington	I	RS49	11.54	Rainbow trout, Virginia	I
RS13	C75-199	Rainbow trout, British Columbia	I	RS50	TH75	Coho salmon, Oregon	I
RS14	ONT347	Rainbow trout, Ontario	II	RS51	11.34	Unknown, Colorado	I
RS20	0634	Brook trout, Australia	I	RS52	11.40	Rainbow trout, North Carolina	I
RS21	0589	Atlantic salmon (?), Australia	I	RS54	YR-40	Dolly Varden trout, British Columbia	I
RS24	2.87	Rainbow trout, Colorado	V	RS55	ONT487	Rainbow trout, Ontario	I
RS25	2.88	Rainbow trout, Colorado	V	RS61	42/81	Unknown, England	I
RS26	126-76	Rainbow trout, Idaho	I	RS62	83-206-1	Whitefish, British Columbia	V
RS27	2#C-M#8	Rainbow trout, Idaho	I	RS63	F.15.1/83	Rainbow trout, West Germany	UT <sup>d</sup>
RS28	9-4-1	Unknown	I	RS64	F.111/81	Rainbow trout, West Germany	I
RS32	9-4-5 <sup>c</sup>	Rainbow trout, Nova Scotia	I	RS66	ONT518	Lake trout, Ontario	II
RS33	YR12	Rainbow trout, New Mexico	I	RS68	83-106-7	Steelhead trout, British Columbia	I
RS34	YR20	Rainbow trout, California	I	RS69	ONT534	Lake trout, Ontario	I
RS35	JW10-76	Unknown (reference strain)	I				
RS36	JW8-78	Unknown (reference strain)	I				
RS37	JW76-78	Rainbow trout, Wisconsin	I				

<sup>a</sup> Brown trout (*Salmo trutta*), rainbow trout (*Salmo gairdnerii*), chinook salmon (*Oncorhynchus tshawytscha*), brook trout (*Salvelinus fontinalis*), cisco (*Coregonus artedii*), Atlantic salmon (*Salmo salar*), coho salmon (*Oncorhynchus kisutch*), Dolly Varden trout (*Salvelinus malma*), whitefish (*Coregonus clupeaformis*), steelhead trout (*Salmo gairdnerii*), lake trout (*Salvelinus namaycush*), muskrat (*Ondatra zibethica*).

<sup>b</sup> Type strain.

<sup>c</sup> Strains RS9 and RS32 were from the same initial source but RS9 appears to have lost a minor antigen after frequent subcultures (14a).

<sup>d</sup> UT, Untyped.

**Electron microscopy.** Phage preparations were centrifuged for 1 h at 70,000 × *g* to increase the particle concentration for electron microscopy. A drop of the phage suspension was placed on a carbon-coated grid for 1 to 2 min and then removed with filter paper, leaving a thin film. The particles were negatively stained with 2% ammonium molybdate (pH 6.8) and examined with a Philips EM 300 electron microscope.

**Host range.** Phage stocks were titrated to determine a suitable routine test dilution (RTD), defined as the highest dilution that produces just-confluent or semiconfluent lysis on the propagating or host strain (1). Duplicate plates with top agar lawns of the strains to be tested were spotted with 0.01-ml drops of the various phage suspensions, incubated overnight at 25°C, and examined. The lytic reaction patterns of the different strains of bacteria were recorded on punch cards, which were sorted manually to identify correlations between phage sensitivities and serological reactions. Bacteriophages showing unique patterns of lysis were selected for further study.

The host ranges of the selected phages were retested against isolates of *Y. ruckeri* and against strains of *Y. enterocolitica* and other members of the family *Enterobacteriaceae* with three different concentrations of phage stocks. Top agar overlay plates of the bacteria were spotted with triplicate 15-μl drops of three different phage dilutions,

generally RTD, 100× RTD, and 1,000× RTD, and incubated at 25°C overnight.

## RESULTS

**Bacteriophage isolations.** Eight sewage enrichments produced areas of lysis or individual plaques when tested on lawns of the homologous *Y. ruckeri* strain. These could be transferred and plaque purified, indicating that they were bacteriophages rather than bacteriocins or other toxic products. The majority of plaques were very small or very turbid (Table 2). In contrast, large clear plaques were obtained with a *Y. enterocolitica* enrichment of the same sewage sample. A mud sample obtained from the outflow pond of an Ontario hatchery and enriched with a culture previously obtained from that source was the source of one bacteriophage. Tests with mitomycin C-induced supernatant fluids or *Y. ruckeri* cultures produced only one apparently lysogenic phage, isolated from plaques on a lawn of RS62 in a region where culture extracts from RS1, RS6, and RS7 had run together. As the phage was not seen in subsequent tests of these culture preparations, its real source is not known.

**Phage characteristics.** Of 18 bacteriophage preparations, 2 lost all plaque-forming activity after 20 days of storage at 4°C. The other phages were still active after 80 days of storage at 4°C, although most showed a 10- to 100-fold loss in infectivity titer (data not shown). When the 16 remaining

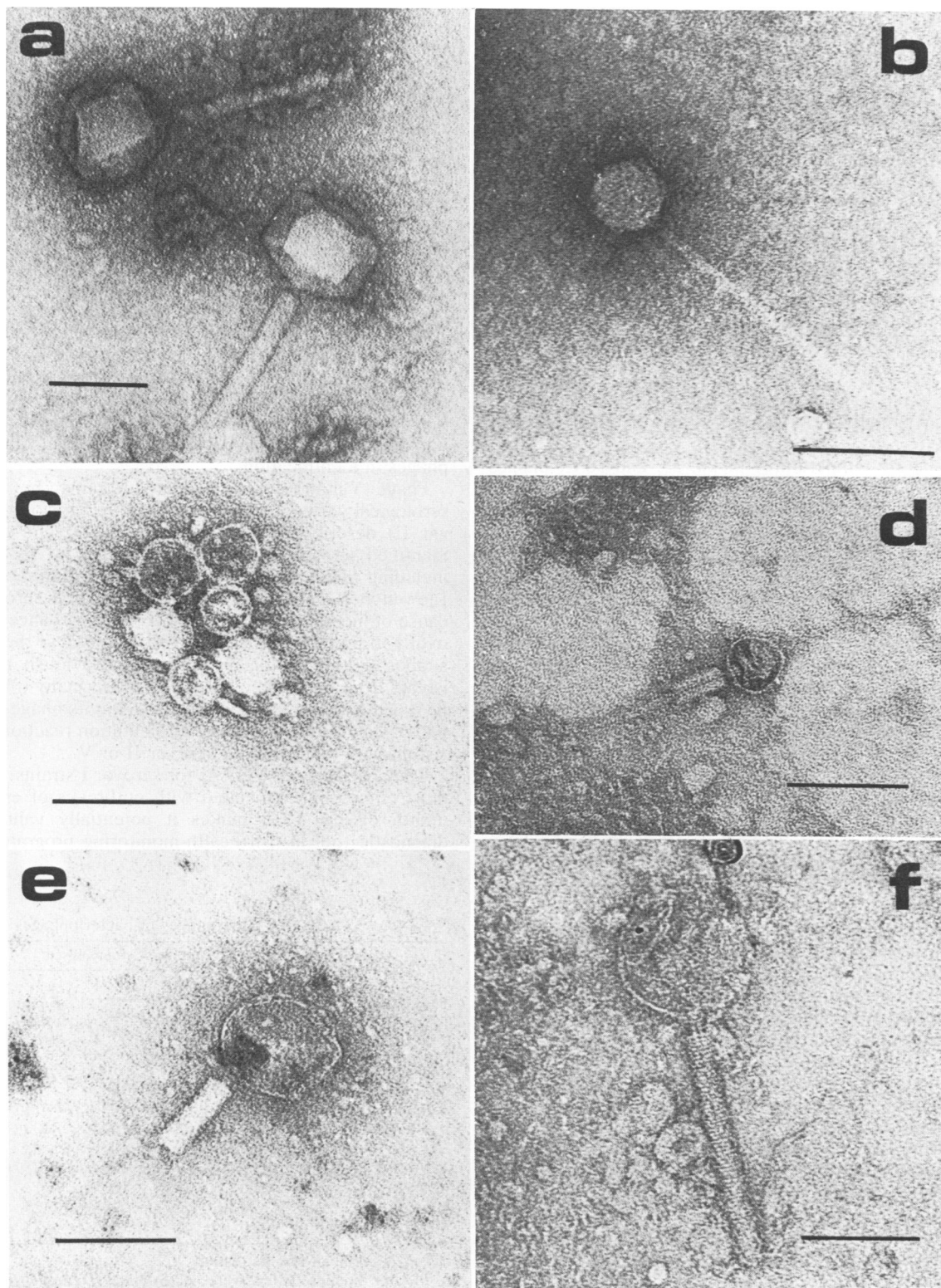


FIG. 1. Electron micrographs of *Y. ruckeri* phages. (a) YerA41, (b) Yer2AT, (c) YerA14, (d) YerA3, (e) YerA20, and (f) YerA7. Bar, 100 nm.

bacteriophages were tested against strains of *Y. ruckeri*, phage YerA41 was of immediate interest because it lysed all strains which had been assigned to serovar I with the exception of one autoagglutinating strain. The sources, host strains, and plaque morphologies of YerA41 and seven other

phages selected for further study are summarized in Table 2.

The majority of the phages were tailed with icosahedral or octahedral heads (Fig. 1). YerA41 appeared to have a contractile sheath and tail, as detached tail components were common in some preparations (data not shown). Phage

TABLE 2. Characteristics of phage isolates

Phage isolate	Strain	Source	Plaque morphology	Particle length (nm)
YerA41	RS41	Sewage	Clear to turbid, 0.7–1.2 mm	300
Yer2AT	RS2	Mud	Turbid, pinpoint	250
YerA7	RS7	Sewage	Turbid, 0.7–1.2 mm	325
YerA14	RS14	Sewage	Turbid, 0.5–1.0 mm	310
YerA3	RS3	Sewage	Turbid, 3.0–4.9 mm	140
YerA10	RS10	Sewage	Foggy, 0.5–1.0 mm	ND <sup>a</sup>
YerA20	RS20	Sewage	Turbid, pinpoint	170
YerL62	RS62	Lysogenic induction	Clear, 0.2–0.5 mm	ND
Yer31B	RS31	Sewage	Clear, 2–3 mm	185

<sup>a</sup> ND, Not done.

YerA14 had a relatively large head with a very short tail (Fig. 1c).

At incubation temperatures of 20, 25, and 30°C, phages YerA41, YerA7, and Yer2AT produced the same degree of lysis, with somewhat more distinct clearing at 30°C. There was less lysis at 15°C and also at 37°C, when the phages were tested against strains able to grow at the higher temperature (4).

**Host ranges.** When tested more stringently, with three different phage concentrations, phage YerA41 remained highly specific for serovar I strains of *Y. ruckeri* (Table 3). It also lysed *Escherichia coli* B, one strain each of *Enterobacter cloacae*, *Erwinia herbicola*, and *Klebsiella pneumoniae*, and four of eight *Y. enterocolitica* strains tested. Phage Yer2AT lysed fewer serovar I strains but almost all of the other serological types tested (Table 3). Phages YerA3, YerA7, YerA10, YerA14, and YerA20 did not differ greatly in the lysis of *Y. ruckeri* strains (Table 3). Phages Yer2AT, YerA3, YerA7, YerA10, YerA14, and YerA20 lysed strains of serovars I', II, and V and many other enteric bacteria (Table 3). Serovar I strains which were sensitive to several of these phages were, in general, sensitive to all of them (Table 4).

In initial trials of host strain specificity, we have observed greater variation in the patterns of hosts lysed by these phages (data not shown). The change may have been due to positive reactions given at phage concentrations higher than the RTDs used in the initial tests. A notable effect of phage concentration on host specificity was seen with phage Yer2AT. Of the 34 strains of *Y. ruckeri* that were lysed by the undiluted phage preparation (10<sup>7</sup> PFU/ml), 10 were lysed by a 10<sup>2</sup> dilution of the preparation, whereas only 2, RS2 and RS14, were lysed by a 10<sup>4</sup> dilution. These strongly reacting bacteria were isolated from fish at the hatchery which was the source of the mud for phage Yer2AT isolation.

The induced phage, YerL62, lysed only its host, RS62. Two other strains assigned to serovar V, RS24 and RS25, were not lysed by YerL62, but at high phage concentrations a few plaques were seen on lawns of two *Y. enterocolitica* strains.

## DISCUSSION

At least four different phages effective against *Y. ruckeri* have now been isolated. YerA41, Yer2AT, and YerL62 clearly differ in host specificities, whereas phages YerA3, YerA7, YerA10, YerA14, and YerA20, which have very similar lysis patterns, represent at least one additional phage. Phages of this last group showed some differences;

for example, YerA3 was much more active against *Y. enterocolitica* strains than were other phages (Table 3), and YerA14 appeared to have a distinctive particle morphology (Fig. 1). Particle sizes and plaque morphologies also differentiate these five phages.

Additional phages with narrower host specificities would be desirable for a phage-typing scheme. *Y. ruckeri* serotypes may be fairly uniform in their phage receptor sites, but the very narrow host range of YerL62 and the preferential activity of Yer2AT against bacteria from the same source argue against this. We had limited success at obtaining phages by induction, which may indicate an infrequent incidence of lysogenic strains of *Y. ruckeri*.

Only 2 of the 51 strains, RS1 and RS6, failed to react with any of the phages (Table 4). Strain RS1 had been thought to be of a distinct serological group (serovar IV) (14a). Its nonreactivity with *Y. ruckeri* phages is consistent with our recent DNA-DNA hybridization results, which suggest that RS1 is in fact not a strain of *Y. ruckeri* (S. A. De Grandis, unpublished data), and illustrates the potential value of these phages in identification.

Phage YerA41 lysed serovar I strains, but not such serological varieties of *Y. ruckeri* as the Oregon type (serovar II) described by O'Leary et al. (14) and our newly identified serovar V strains (14a) (Table 3). Three strains, including the salmonid blood spot bacterium described by Llewellyn (11), were previously designated serovar I' because of incomplete serological cross-absorbances with serovar I strains (14a). Phage YerA41 did not lyse these strains, supporting the serological distinction previously made. One isolate from the Federal Republic of Germany (8) could not be typed with our standard antisera, but its phage sensitivity pattern and positive sorbitol fermentation reaction was consistent with that of either serovar II or V.

The specificity of YerA41 for serovar I strains, which are most commonly associated with outbreaks of enteric redmouth disease (12), makes it potentially valuable as a diagnostic tool in fish health-monitoring programs. It pro-

TABLE 3. Host ranges of bacteriophages

Phage type	Percentage of strains lysed <sup>a</sup>					Other species (n = 27) <sup>b</sup>
	<i>Y. ruckeri</i> serovar:				<i>Y. enterocolitica</i> (n = 8)	
	I (n = 35)	I' (n = 3)	II (n = 8)	V (n = 3)		
YerA41	97	0	0	0	50	15
Yer2AT	63	100	88	100	38	48
YerA7	51	100	88	100	13	30
YerA14	60	100	88	100	100	30
YerA3	63	100	88	100	88	56
YerA10	60	100	88	100	25	33
YerA20	60	100	88	100	13	67
YerL62	0	0	0	33	25	0
Yer31B	0	0	0	0	75	26

<sup>a</sup> n, Number of strains tested.

<sup>b</sup> Other species tested were: *Citrobacter freundii*, *Edwardsiella tarda*, *Enterobacter aerogenes*, *Enterobacter agglomerans*, *Enterobacter cloacae* (two strains), *Erwinia herbicola*, *Escherichia coli* (three strains), *Hafnia alvei* (two strains), *Klebsiella pneumoniae*, *Proteus mirabilis*, *Proteus morgani*, *Proteus rettgeri*, *Proteus vulgaris*, *Providencia stuartii*, *Salmonella arizonae*, *Salmonella cholerae-suis*, *Salmonella pullorum*, *Salmonella typhimurium*, *Serratia liquefaciens*, *Serratia marcescens*, *Shigella boydii*, *Shigella flexneri*, and *Shigella sonnei*. All cultures were from the Department of Microbiology, University of Guelph.

TABLE 4. Patterns of lysis for *Y. ruckeri* strains

<i>Y. ruckeri</i> group	Lytic activity of phage: <sup>a</sup>							RS strains in group (laboratory code no.)
	YerA41	Yer2AT	YerA7	YerA14	YerA3	YerA10	YerA20	
Serovar I								
a	+	-	-	-	-	-	-	4, 11, <sup>b</sup> 12, 13, 27, 33, 34, 41, 43, 46, 47, 48, 52, 61
b	+	+	-	-	-	-	-	5, 9, 26, 28, 32, 35, 36, 37, 38, 40, 42, 51
c	+	+	-	-	+	-	-	68
d	+	+	-	+	+	+	+	45, 48, 50
e	+	+	+	+	+	+	+	7, 55, 64, 69
f	-	-	-	-	-	-	-	39 <sup>c</sup>
Serovar I' (g)	-	+	+	+	+	+	+	20, 21, 54
Serovar II (h)	-	+	+	+	+	+	+	2, 3, 8, 10, 14, 44, 66, 67
Serovar V (i)	-	+	+	+	+	+	+	24, 25, 62

<sup>a</sup> +, Lysis; -, no reaction.

<sup>b</sup> Type strain.

<sup>c</sup> Autoagglutinating strain (14a).

vides a means of confirming the slide agglutination test recommended by the American Fisheries Society (13), and it may be possible to develop a rapid screening phage test such as that used for salmonellae detection (7). The effect of YerA41 on other bacterial species forming the normal microbiota of fish will need to be more critically assessed before such an application. Cross-reactions of the phages isolated from sewage enrichments with different members of the *Enterobacteriaceae* are perhaps not unexpected. Although Dudley et al. (5) reported a *Y. ruckeri* isolation from sewage sludge, our phages may well have had a different original host bacterium.

We were interested in determining whether variations in phage-sensitivity patterns observed among the 35 serovar I strains (Table 4) could be correlated with other characteristics, such as plasmid profiles or the ability to grow at both 37 and 25°C (4). The seven strains which are sensitive both to YerA41 and to several of the group YerA3, YerA7, YerA10, YerA14, and YerA20 are all capable of growth at 37°C (4; S. A. De Grandis, unpublished observations). Kawaoka et al. (9) have described growth temperature-dependent differences in bacteriophage receptors of *Y. enterocolitica*, and we speculate that a similar cell-surface difference could account for the behavior of these *Y. ruckeri* strains with respect to both growth temperature and phage sensitivity. The bacteriophages have potential value in further studies of strain variations.

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